Body composition and metabolic profile of children with end stage liver disease

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BODY COMPOSITION AND METABOLIC PROFILE OF CHILDREN WITH END STAGE LIVER DISEASE

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Abstract

Children with end stage liver disease are thought to have reduced lean mass and higher energy requirements. The aim of the study was to assess how body composition and energy requirements of children with end stage liver disease differ from those of healthy children and how they change after liver transplantation as well as to study for metabolic pathways that may be influencing the body composition.

Body composition was assessed by various methods including basic anthropometry, stable isotopes, dual-energy X-ray absorptiometry (DXA) and air displacement plethysmography (BOD POD). Resting energy expenditure was assessed by indirect calorimetry. The patients were compared to age and sex matched healthy controls and were re-assessed at least 6 months after they received their liver transplant. Liver, muscle and fat tissue obtained at the time of transplant was assessed by microarray analysis of gene expression and validated with qPCR with a focus on metabolic pathways of glycolysis, lipolysis, insulin resistance and muscle atrophy and in particular the AMPK pathway (a cellular catabolic pathway).

Seventeen patients and 14 healthy controls were recruited. Basic anthropometry showed that the patients had significantly lower weight and height. The deuterium, BOD POD and DXA measurements showed that whereas some children had lower lean mass, fat mass (FM) was preserved and remained within the normal range. Body mass index, mid upper arm circumference and FM indices were negatively correlated with stay in hospital post liver transplant. The presence of hypermetabolism was not different between patients and controls. The tissue studies distinguished a subgroup of patients with significant differences in their muscle and fat tissue. These differences were not related to catabolic pathways including AMPK, but were related to a pathway linked to inflammatory mediated insulin resistance involving interleukin-6.

In this cohort of patients, in spite of lean mass reduction, fat mass was preserved and correlated with a shorter stay in hospital after liver transplantation. This is relevant information when deciding on appropriate nutritional management prior to transplantation. Some patients may develop insulin resistance as a mechanism of fat mass preservation during their chronic illness. This is the first time a possible mechanism for the insulin resistance described in these patients is identified. Further work would be required to confirm this finding and to link it with clinical evidence of insulin resistance.

Table of Contents

Table of Figures

Table of Tables

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Abbreviations

4E-BP1	4E- binding protein 1
ACC	Acetyl- CoA carboxylase
ACR	Acute cellular rejection
ACTH	Adrenocorticotrophic hormone
ActRIIB	Activin receptor IIB
AgRP	Agouti related peptide expressing
Akt	Protein kinase b
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANS	Autonomic nervous system
AP-1	Activator protein 1
AST	Aspartate transaminase
AST	Aspartate aminotransferase
ATGL	Adipocyte triglyceride lipase
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
Atrogin-1	same as MAFbx
BA	Biliary atresia
BAT	Brown adipose tissue
BCAA	Branched chain amino acids
BCM	Body cell mass
BIA	Bioelectrical impedance
BMC	Body mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP7	Bone morphogenetic protein 7
BMR	Basic metabolic rate
BW	Body weight
CART	Cocaine and amphetamine regulated transcript expressing
CBS	Corrected bromide space
CCL2	C-C Motif chemokine ligand 2
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CN	Crigler-Najjar type 1
CNS	Central nervous system
CO_2	Carbon dioxide
CRH	Corticotrophin releasing hormone
CRP	C reactive protein
CT	Computed tomography
DXA	Dual-energy X-ray absorptiometry
ECW	Extra cellular water
eIF4E	Eukaryotic initiation factor 4E
ERK	Extracellular signal regulated kinase
ESCLD	End stage chronic liver disease
FBC	Full blood count
Fbxo40	F-box protein 40
FFM	Fat free mass
FM	Fat mass

FMI	Fat mass index
FoxO	Forkhead box O
GDF8	Growth differentiation factor 8
GGT	Gamma glutaryl transferase
GGT	Gamma glutamyl transpeptidase
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GLUT4	Glucose transporter 4
GSK3β	Glycogen synthase kinase β
HAT	Hepatic artery thrombosis
HB	Haemoglobin
HCC	Hepatocellular carcinoma
HMG-CoA reductase	3-- Hydroxyl 3-methylglutaryl- coenzyme A reductase
HSL	Hormone sensitive lipase
ICP-MS	Inductively coupled plasma mass spectometry
ICU	Intensive care unit
ICV	Intracerebroventricular
ICW	Intracellular water
IGF-1	Insulin-like growth factor 1
IL 1β	Interleukin 1β
IL6	Interleukin 6
IL8	Interleukin 8
IM	Illness marker
INR	International normalised ratio
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
LCT	Long chain triglycerides
LFTs	Liver function tests
LKB1	Liver kinase B1
LM	Lean mass
LMI	Lean mass index
MAFbx	Muscle atrophy F box
MAG	Monoacylglycerol
MAPK	Mitogen activated protein kinase
MC4R	Melanocortin receptors type 4
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCT	Medium chain triglycerides
MCV	Mean corpuscular volume
mTOR	Mammalian target of rapamycin
mURF-1	Muscle RING finger- 1
Na	Sodium
NaBr	Sodium bromide
NEFAs	Non esterified fatty acids
NFκβ	Nuclear factor κβ
NPY	Neuropeptide Y
NSC	Neonatal sclerosing cholangitis
O_2	Oxygen
PA	Phase angle
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase

PLIN	Perilipin
PLTs	Platelets
POMC	Proopiomelanocortin
PSC	Primary sclerosing cholangitis
PTLD	Post-transplant lymphoproliferative disease
qRT-PCR	Relative quantification real time PCR
R	Resistance
REE	Resting energy expenditure
S6K1	Ribosomal protein S6 kinase beta-1
SAA	Serum amyloid A
SD	Standard deviation
sds	Standard deviation score otherwise known as z score
SNS	Sympathetic nervous system
SOCS3	Suppressor of cytokine signalling 3
TBA	Total bile acids
TBW	Total body water
TEE	Total energy expenditure
TFT	Thyroid function tests
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor a
TRH	Thyrotropin releasing hormone
U+Es	Urea and electrolytes
UCP 1	Uncoupling protein 1
UPS	Ubiquitin- proteasome system
WAT	White adipose tissue
WCC	White cell count
Xc	Reactance
Z	Impedance

Chapter 1 INTRODUCTION

End stage chronic liver disease (ESCLD) has been defined as “the consequence of many chronic liver diseases, leading to irreversible impairment of liver function, architecture (cirrhosis) and blood supply” (Sokal et al., 2008). Liver transplantation is the only realistic treatment option for ESCLD. The liver is the most metabolically active organ which interacts with muscle and fat tissue via processes like gluconeogenesis, glycogenolysis, fatty acid oxidation, synthesis of lipoproteins, protein synthesis and protein degradation. In spite of being only 1.5-2% of adult body weight, 20%-25% of our resting energy expenditure can be attributed to the liver (Muller, 1998). ESCLD is known to affect body composition (Sinclair et al., 2016, Maharshi et al., 2015, Peng et al., 2007).

Body composition in end stage chronic liver disease.

1.1.1 Body composition of adults with ESCLD

Patients with chronic liver disease and cirrhosis are known to suffer from protein- energy malnutrition (Lautz et al., 1992, Maharshi et al., 2015, Thandassery and Montano-Loza, 2016). This comes as a consequence of various factors. The most important are anorexia and fat malabsorption (Romiti et al., 1990, Beckett et al., 1980) due to cholestasis. Additional factors are prescribed low salt diets and micronutrient deficiencies contributing to dysgeusia (Reifen et al., 1998, Heckmann et al., 2005). The presence of organomegaly and ascites further contribute to feelings of fullness and discomfort whilst eating.

Multiple studies in adults with cirrhosis have shown significant changes to body composition when compared to healthy controls (Peng et al., 2007, Vulcano et al., 2013, Riggio et al., 1997). These changes are reduction of lean mass (mainly skeletal muscle), reduction of total body fat, reduction of bone mineral density and an increase in extra cellular water. In addition these patients have been shown to have abnormal metabolic profiles with hypermetabolism, insulin resistance and abnormal protein metabolism (Kondrup and Muller, 1997, Selberg et al., 1993, Muller et al., 1999, Petrides et al., 1991).

The reduction of skeletal mass is called sarcopenia. Sarcopenia has been described in male and female patients with cirrhosis, but tends to be more common in males (Riggio et al., 1997, Kim and Jang, 2015, Montano-Loza et al., 2012). Sarcopenia has been linked to worse outcomes both before (Montano-Loza et al., 2012, Selberg et al., 1997) and after liver

transplantation (Dimartini et al., 2013, Montano-Loza, 2014). Studies have shown that the presence of sarcopenia does not necessarily correlate with the disease severity (Figueiredo et al., 2005, Riggio et al., 2003, Hara et al., 2016) and may be present from the early stages of cirrhosis.

Sarcopenic obesity is the term used to describe the presence of sarcopenia in an obese individual (Roubenoff, 2004). Two patients with the same body mass index (BMI) may have significantly different body composition. As obesity becomes more prevalent in our society, the risk of not identifying sarcopenia in patients with liver disease who could be obese increases. One study reported 56% of obese (BMI ≥ 30) cirrhotic patients to fulfil the criteria of muscle wasting on computed tomography (CT) as per established definitions for cancer patients (Cruz et al., 2013).

Sarcopenia is important because it has been repeatedly shown to be associated with an increase in morbidity and mortality both before (Selberg and Selberg, 2002, Tandon et al., 2012, Montano-Loza et al., 2012) and after (Hamaguchi et al., 2014, Masuda et al., 2014, Kaido et al., 2013, Englesbe et al., 2010, Tsien et al., 2014) liver transplantation. Montano-Loza et al (Montano-Loza et al., 2012) demonstrated that the presence of sarcopenia significantly reduces the median survival time before liver transplant from 34 months to 19 months and the presence of sarcopenia did not correlate with the severity of the liver disease. Further to this Tsien et al showed that after liver transplantation in a cohort of 53 patients sarcopenia increased from 66.2% to 88.6% and was associated with an increase in mortality. The patients in this study were male and a significant proportion were receiving the transplant for hepatocellular carcinoma (HCC), nevertheless the study does raise awareness of the potential increase of sarcopenia post liver transplant.

Loss of body fat is observed in patients with cirrhosis. Some studies report a more pronounced loss of body fat in the initial stages of the liver disease which is then followed by an increase in loss of body cell mass in the later stages regardless of the patient's gender (Figueiredo et al., 2005). Other studies claim that body fat is reduced in women with cirrhosis and that lean tissue is preserved, whereas men show a reduction in both lean and fat tissue (Riggio et al., 2003). These differences between the genders may reflect the different body composition make up of men and women; men naturally have more muscle and women have more fat. The variation in findings also has to do with the different study populations. In Riggio's study, patients with HCC were excluded, so the study population suffered purely from cirrhosis. The patients were stable

cirrhotics, which was defined as patients with less than 5% of weight loss in the preceding 6 months. In the Figueiredo study the patients had various diagnoses and were at different stages of disease.

Liver disease is known to affect bone health. Patients with liver disease may suffer from hepatic osteodystrophy, a term used to describe the clinical manifestations of the metabolic bone disease. Pathogenesis appears to be diverse e.g. deficiency in 25-hydroxylation of vitamin D by the liver resulting in secondary hyperparathyroidism, deficiency of vitamin K required for making bone matrix protein, insulin growth factor 1 (IGF-1) deficiency or insensitivity (which is involved in osteoblast differentiation and proliferation), hypogonadism, medications particularly steroids, a sedentary lifestyle and poor nutrition (Lopez-Larramona et al., 2011, Stokes et al., 2013, Soylu et al., 2012). The prevalence of osteoporosis in adults with advanced liver disease is reported at 12-55% (Gatta et al., 2014) and again there is variation in the patterns of bone density loss reported. Some studies report a loss in bone mineral content (BMC) in females, but not in males (Figueiredo et al., 2005), whereas others report loss in bone mineral density (BMD) and BMC in male and females (Riggio et al., 1997). One cross sectional study that has looked at BMC before and after liver transplant, found no changes overall (Meys et al., 1994), but when they followed the same patients before and after transplant they found a 3.5% reduction in BMC after liver transplantation, as also reported by Gatta et al (Gatta et al., 2014).

Fluid retention presenting as ascites or as whole body fluid retention and oedema is a common complication of ESCLD. Cirrhosis results in portal hypertension and splachnic vascular and peripheral arterial vasodilation thought to be due to an overproduction of nitric oxide. This then results in a reduction of effective cardiac output and an increase in antidiuretic hormone, stimulation of the sympathetic nervous system (SNS) and the renin- angiotensin-aldosterone system, all leading to sodium and water retention (Kashani et al., 2008). Water retention is the main reason that makes basic anthropometry very difficult to use in these patients. Apart from an increase in their total body water, patients with cirrhosis exhibit a redistribution of their body water, with an increase in extracellular water (ECW) and a reduction in intracellular water (ICW) even before oedema is clinically evident (Figueiredo et al., 2005, Lehnert et al., 2001).

Hypermetabolism has frequently been described in patients with cirrhosis. It is usually defined as an increase of the resting energy expenditure (REE) of the patient to over 120% of the predicted REE, as calculated by relevant equations or when compared to appropriately matched controls (Muller et al., 1999, Ferreira et al., 2013). Basic metabolic rate (BMR) is the heat

production of the individual in a state of complete muscular repose 12-24 hours after the last meal, i.e. in the post-absorptive condition. Resting energy expenditure is the energy required by the body at rest, as measured in less strict conditions to BMR e.g. less hours of fasting. Only about 15-31% of patients are hypermetabolic (Muller et al., 1994b, Selberg et al., 1997) and some patients are in fact hypometabolic. Hypermetabolism does not seem to be associated with particular clinical or biochemical measures, but it has been linked to worse outcomes mainly before liver transplantation. Fewer studies have looked at hypermetabolism after transplantation particularly after the first 6 months. The studies that have looked into it show a persistence of hypermetabolism after liver transplantation and an association with poorer liver function (Muller et al., 1994b).

Not all studies confirm the presence of hypermetabolism and some argue that, because total energy expenditure in cirrhotic patients is either normal or reduced (Riggio et al., 2003), possibly because of reduced physical activity, even if there was an increase in REE it would not be of clinical significance. Studies that have looked at energy intake and energy balance in patients with cirrhosis show that these patients do balance their expenditure with their intake, but that their expenditure is reduced in comparison to the healthy population allowing therefore for nutritional rehabilitation (Nielsen et al., 1993). Patients with HCC are generally accepted to be hypermetabolic (Chen and Chung, 1994) and therefore care needs to be taken when reviewing the literature that studies looking into energy expenditure take this into account.

The reason hypermetabolism is interesting in cirrhosis, and indeed in other patients with chronic disease, is because it represents a maladaptation of the body to the reduction in caloric intake/ caloric absorption. In starvation and in anorexia nervosa people tend to reduce their REE, something which is not the case in these chronically ill patients (Muller et al., 1994a).This hypermetabolism, or even surprisingly ‘normal’ REE implies that there are other metabolic disturbances inherent to the disease process influencing the metabolic rate. The variance in REE is influenced by body cell mass, age, sex and genetic make-up (Ravussin and Bogardus, 1989). There is some experimental evidence to show that as portal venous flow to the liver reduces, hepatic energy expenditure reduces as well, but at the same time whole body energy expenditure increases (Muller et al., 1994a). Linking this to what was previously mentioned when discussing fluid retention in chronic liver disease, it may be that the increase in cardiac output and sympathetic nervous system (SNS) stimulation may explain the increase in whole body energy expenditure.

Apart from aberrations in energy metabolism, aberrations are noted in substrate metabolism. Cirrhotic patients have been shown consistently to be insulin resistant (Selberg et al., 1993, Petrides et al., 1991). The insulin resistance has been shown to be a reflection of reduced glycogen synthesis and reduced insulin dependent glucose transport into the muscle cells (Selberg et al., 1993, Petrides et al., 1991) and not due to hepatic insulin resistance. Even though fat intestinal absorption, particularly in cholestatic patients, is reduced, fat metabolism has been shown to be normal (Kondrup and Muller, 1997). The patients rely on fat oxidation for energy, most likely because glycogen levels in the liver are depleted (Muller et al., 1992a, Greco et al., 1998). Protein requirements for patients with liver disease are at least that of normal individuals or higher, in contrast to malnourished people without underlying disease, who have reduced protein requirements (Nielsen et al., 1995). To try and understand the reason for this, one study compared cirrhotic patients with an increased protein requirement to those with a normal protein requirement. It found that the patients with the increased protein requirements had an increase in protein degradation, perhaps due to reduced insulin-like growth factor 1 (IGF-1) levels (Kondrup et al., 1997).

IGF-1 is the main mediator of the effects of growth hormone (GH) and is exclusively made in the liver. It is therefore not surprising that circulating IGF-1 levels are low in liver disease (De Palo et al., 2001, Yoshida et al., 2003). Whereas the liver and fat cells only have insulin receptors, muscle cells have insulin and IGF-1 receptors. IGF-1 in muscle increases the uptake of amino acids and protein synthesis in muscle. Therefore low IGF-1 levels could contribute to the mechanism of muscle wasting seen in chronic liver disease. In recent years many more hormones and their role have been studied in liver disease. Particular interest has been aimed at adipokines like leptin and adiponectin. Leptin has been noted to be reduced in end stage liver disease and serum levels of adiponectin have consistently been noted to correlate with severity of liver fibrosis (Kalafateli et al., 2015, Roberts et al., 1998, Dornelles et al., 2013).

End stage chronic liver disease can be effectively managed with liver transplantation. This comes with its own complexities, but it does achieve excellent outcomes for the patients with very good post-transplant survival rates. Increasingly studies are looking into the effect of liver transplantation on body composition of patients. One study demonstrated, at a median of 2 years post liver transplant, a prevalence of sarcopenic obesity at 88% with half of the patients fulfilling the criteria of metabolic syndrome (Choudhary et al., 2015) and, as mentioned earlier, sarcopenia seems to remain a feature post liver transplant as well. Another study measured

with dual-energy X-ray absorptiometry (DXA) an increase of 4.75% in fat mass a year after liver transplant (Meys et al., 1994). These are all important findings. Previously it was thought that as the liver disease is 'removed' with the change of the native liver, the nutritional and metabolic issues would also resolve. As liver transplant recipients enjoy an increase in survival, it becomes more important to know how their body composition is affected and what implication this has for their long term health and well-being.

1.1.2 Body composition of children with ESCLD

Studies in children with liver disease are limited, but they also indicate alterations in body composition. Studying body composition in children is challenging as the various measurements have to be adjusted for age and the availability of normal data for comparison is not always possible. Particularly in the context of liver disease it has long been established that due to organomegaly and the possible presence of fluid retention and ascites, as with adults so with children, basic anthropometry measures like weight underestimate the presence of malnutrition (Trocki et al., 2000).

Greer et al (Greer et al., 2003) studied 21 children with end stage liver disease awaiting liver transplantation and compared them to 15 age and sex matched controls. These children were found to have a reduction in total body fat, an increase in extracellular water, but no significant difference in body cell mass (muscle and solid organ mass), as measured by total body potassium. The percentage of body cell mass per body weight was increased. REE, when expressed per body weight, was higher than in controls by 27%, presumably because a higher percentage of this body weight was now active muscle and solid organ mass rather than fat tissue. When REE was expressed per body cell mass it was again increased in comparison to healthy controls by 16%. This could be explained if there was a reduction in the body cell mass (BCM), but this was not seen in this study. Alternatively the increase in REE/BCM could be because of a change in energy metabolism related to the disease itself. In Greer's study only average REE between the two groups is reported, but looking at the range of the values for REE there is overlap between the patients and the controls. This implies that even though on average the patients had a higher REE than the controls, this is not necessarily the case at an individual level for all the patients. The study of Chin et al of 27 children with end stage liver disease did demonstrate a reduction in body cell mass, as total body potassium of the patients was 63% of the expected for age and sex (Chin et al., 1992). An older study of 11 infants with

extra hepatic biliary atresia by Pierro et al has demonstrated a 29% higher REE than expected for weight (Pierro et al., 1989).

At this point a particular group of patients with chronic liver disease needs to be mentioned, it is that of children with Alagille syndrome. This syndrome is genetically inherited and growth faltering has been described even when these patients are compared to patients with biliary atresia. They tend to be stunted with lower weight and lower fat and muscle mass (Arvay et al., 2005). They have not been shown to be hypermetabolic (Rovner et al., 2006).

After paediatric liver transplantation it has been shown that weight gain and appropriate growth is usually achieved, even though it may take a few years before height standard deviation (sd, or otherwise known as z) scores catch up (Holt et al., 1997, van Mourik et al., 2000). Poor graft function is associated with poor growth post-transplant (Holt et al., 1997), but it is not known how this relates to pre transplant body composition and energy metabolism. Increasingly studies are showing that after transplantation some patients accumulate fat e.g. after haematopoietic stem cell transplant (HSCT) in childhood 15.6% of patients develop insulin resistance and central fat adiposity in adulthood (Bizzarri et al., 2015). A recent study that has looked at BCM of children before and after liver transplantation (median time post-transplant 7.23 years) found that even though weight had increased and was now in the normal range, there was no increase in BCM (Ee et al., 2014), indicating that the catch up in weight was due to an increase in fat mass. To our knowledge, no studies so far have looked at REE after liver transplant in children.

In summary, faltering of growth has been described in children with chronic liver disease with various changes in fat tissue and lean mass. Most previous studies have suggested that children with chronic liver disease are hypermetabolic. There have been some data to suggest that after liver transplant weight gain reflects an increase in fat tissue and lean mass is not replenished.

Cachexia

1.1.3 Definition of cachexia

This change in body composition, as previously indicated, can be explained to a degree by malnutrition. Malnutrition implies that by correcting the caloric and nutrient insufficiency body composition will return to normal. It is now known from observations and studies in a variety of

chronic diseases that there is another process occurring, frequently with malnutrition, a process termed cachexia. Cachexia, is "*a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with wasting disease. Wasting disease is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism, and is associated with increased morbidity*" (Evans et al., 2008). The hallmark of cachexia is that the muscle wasting cannot be fully reversed by supplementation of calories (Casaer, 2015, Ries et al., 2012, Fearon et al., 2011). Cachexia, in the context of cancer, has been further classified as pre-cachexia (weight loss ≤ 5%), cachexia (weight loss > 5%) and refractory cachexia (Fearon et al., 2011). It may be that pre- cachexia is the stage where most interventions would be most effective.

A more practical consensus definition of cachexia has been reached to help physicians diagnose the condition and also to help for recruitment to studies. In adults, cachexia can be diagnosed if there has been at least 5% weight loss in the last 12 months or less (or the BMI < 20 kg/m^2) and at least 3 of the following 5 conditions are met: presence of decreased muscle strength, fatigue, anorexia, low fat free mass index (mid upper arm circumference (MUAC) < 10th centile for age and sex, appendicle skeletal mass index by DXA in kg/m^2 under 5.45 in females and 7.25 in males) and/or abnormal biochemistry e.g. increased inflammatory markers like C-reactive protein (CRP) and interleukin 6 (IL6), anaemia (haemoglobin (Hb) < 12 g/dL) and low serum albumin (< 32 g/dL) (Evans et al., 2008). In addition various studies have helped create cut offs for what can be considered normal lean mass on imaging for men and women (Kazemi-Bajestani et al., 2016). Unfortunately for children there are no established criteria.

Growth is an essential consideration in defining cachexia for children. Growth parameters in children are usually expressed in centiles or standard deviation scores (known as sd or z scores) to allow comparison between different age groups. Looking in the literature various cut offs have been used for children e.g. failure of linear growth, BMI < 5th centile, body fat % less than 10% or a minimum 5% weight loss from the highest previous weight or an ideal weight for height equal or under 90% (Cuvelier et al., 2014, Mak et al., 2012).

Defining cachexia is important for its diagnosis in the clinical setting and for deciding inclusion criteria of patients in the research setting. A plethora of studies have shown the presence of

cachexia to be linked to worse outcomes for many chronic diseases (Narumi et al., 2015, Celli et al., 2008, Fearon et al., 2006) and because of this cachexia is being studied in cancer, chronic heart failure, end stage renal disease, chronic obstructive pulmonary disease and many other diseases. A lot of effort has been dedicated to delineating the molecular mechanisms of cachexia the last two decades.

1.1.4 Molecular mechanisms of cachexia

The molecular mechanisms of cachexia are complex and not fully understood. These mechanisms involve the central nervous system (CNS) and in particular the hypothalamus, the muscle tissue, the fat tissue and the communications between these systems with the autonomic nervous system, the hormones and the cytokines of the inflammatory cascade (Mendes et al., 2015, Schiaffino et al., 2013, Bing and Trayhurn, 2009, Onesti and Guttridge, 2014).

1.1.4.1 The Central Nervous System (CNS) in cachexia

The brain controls body homeostasis mainly through the hypothalamic melanocortin system (Garfield et al., 2009). Anorexia, reduced appetite, is one of the hallmarks of cachexia. In the hypothalamus reside neurons that directly affect appetite. Pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript expressing (CART) neurons decrease appetite whilst Agouti related peptide expressing (AgRP) and neuropeptide Y (NPY) neurons increase appetite. Proopiomelanocortin (POMC) neurons produce α-melanocyte-stimulating hormone (α-MSH) which stimulates the type 4 melanocortin receptors (MC4R) (Tao, 2010) with anorexigenic actions whilst AgRP acts as an inverse agonist of the MC4R (Tolle and Low, 2008). This system integrates local and systemic signals of energy status in health and illness. During illness the body has to adjust its metabolic processes to meet the energy demand of the acute phase response. As part of this adjustment, particularly when it is dealing with chronic inflammatory conditions, it may end up in a catabolic state with lipolysis of adipose tissue and muscle break down. The pro-inflammatory cytokines tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β) have been shown to have increased levels of expression in the hypothalamus in models of cachexia. When given via intra-cerebroventricular (ICV) injection they can induce skeletal muscle wasting (DeBoer et al., 2009, Arruda et al., 2010). Other cytokines have been less studied and e.g. IL6 seems to have a more elusive role. IL6 expression increases in the hypothalamus during inflammation, but it is not known how it affects muscle mass at the same

time particularly as in the periphery it has been shown to be involved in muscle wasting as well as muscle recovery and regeneration (Fuchs et al., 2013, He et al., 2009, Toth et al., 2011).

The neuroendocrine system is another way the brain participates in whole body energy balance and metabolism. The hypothalamus, in response to stress, produces corticotrophin releasing hormone (CRH), which then increases production of adrenocorticotrophic hormone (ACTH) leading to release of cortisol by the adrenals. Increased cortisol levels have been described in cachexia (Tzanis et al., 2014) and cortisol can directly affect muscle break down and lipolysis. The hypothalamus also controls release of the thyroid hormones via the release of thyrotropin releasing hormone (TRH). TRH increases in the hypothalamus after injection of TNFα in the hypothalamus (Arruda et al., 2010), but peripheral increase of thyroid hormones have not been confirmed in patients with cachexia (Creutzberg and Casaburi, 2003). The hypothalamus also participates in the GH/IGF-1 axis by producing growth hormone releasing hormone (GHRH). Most studies report low IGF-1 levels in cachexia and a peripheral resistance to GH (Anker et al., 2001, Jenkins and Ross, 1996). Last but not least of the hormones controlled by the hypothalamus are the sex hormones and as previously mentioned hypogonadism is a key feature of cachexia for both men and women and in men could be one of the contributing factors to muscle loss (Vigano et al., 2010). Other hormones that feed into the hypothalamus are leptin and ghrelin. In cachectic patients leptin levels have been found to be decreased and ghrelin levels increased. However energy intake does not increase as one would expect, suggesting an altered hypothalamic response to these hormones (Itoh et al., 2004, Xin et al., 2009).

The melanocortin system also mediates the anorexigenic effects of serotonin. Serotonin-induced hypophagia requires MC4R, with no effect seen in MC4R knockout mice (Heisler et al., 2006). Increased levels of tryptophan, the precursor of serotonin, have been found in the plasma and cerebrospinal fluid of patients with anorexia, cancer cachexia and liver cirrhosis (Cangiano et al., 1990, Laviano et al., 1997). Tryptophan metabolism is profoundly affected by inflammatory mediators (Freitas et al., 2001).

In more recent years interest has shifted to how these inflammatory signals are communicated to the hypothalamus and how signals of acute inflammation transition to chronic inflammation with the involvement of non-neuronal cells. Microglia, the macrophages of the CNS, are now thought to be activated by the cytokines and then in turn produce more cytokines and chemokines that then affect the neurons. In a similar way, the endothelial cells of the CNS

vessels, in response to peripheral cytokines, produce more cytokines and also cell adhesion molecules (selectins) for the binding of leucocytes (via their integrins), as well as chemokines like C-C motif chemokine ligand 2 (CCL2) for the attraction of leucocytes. This process further generates cytokines that bind to neuronal receptors (Molfino et al., 2015, Burfeind et al., 2016)

The other way the CNS communicates with the periphery is with the autonomic nervous system (ANS). Through vagal afferents to the brainstem (Appleyard et al., 2005), in a rat model of cancer-induced anorexia, autonomic relay brainstem regions are activated independently of any increase in systemic cytokines (Ruud and Blomqvist, 2007). Intraperitoneal lipopolysaccharide administration activates adrenergic and noradrenergic neurons of the nucleus tractus solitarius (Gaykema et al., 2009), suggesting vagal input perhaps secondary to local inflammation (Laviano et al., 2008, Tracey, 2007). Via the SNS it can directly affect substrate metabolism and influence triglyceride synthesis and white adipose tissue fat accumulation, as well as glucose homeostasis (Rossi et al., 2011, Parton et al., 2007). It also regulates the activity of brown adipose tissue (BAT), which has an important role in thermogenesis (Cypess et al., 2009). BAT is now thought to have a functional role in adults and not just in neonates as previously thought. Retrograde tracers have demonstrated connections between BAT and nuclei involved in energy homeostasis many of which express MC4R (Voss-Andreae et al., 2007). ICV administration of melanocortin agonists increases BAT thermogenesis and lipolysis of white adipose tissue (Brito et al., 2007). BAT is emerging as a key player not only for the development of cachexia and hypermetabolism, but also as a counter balance and potential therapeutic target for the overcoming of obesity.

In summary, the hypothalamus is key to whole body energy and metabolic control. It is affected by acute and chronic inflammatory signals from the periphery during illness and it orchestrates its response via the ANS and the endocrine system. In this way it instigates skeletal muscle proteolysis and adipose tissue lipolysis in an effort to provide for the body a valuable source of amino acids and energy respectively to fuel the acute phase response and the expansion of the immune system required in these situations.

1.1.4.2 Muscle wasting and cachexia

Muscle wasting is one of the hall marks of cachexia (Bowen et al., 2015, Fearon et al., 2011). Muscle wasting results from a change in the balance between protein synthesis/ muscle hypertrophy and protein degradation/ muscle atrophy. Two major pathways are implicated in muscle hypertrophy: the IGF-1/P13K/Akt/mTOR pathway and the myostatin pathway, whilst

three major pathways are implicated in muscle atrophy: the proteasomal pathway (responsible for the degradation of extracellular proteins and cell receptors), the calcium activated pathway (involved in tissue injury, necrosis and autolysis) and the autophagic- lysosomal pathway (which degrades the majority of cellular proteins) (Tisdale, 2009, Lecker et al., 1999, Schiaffino et al., 2013). Each of these pathways are implicated in cachexia (Figure 1.1).

In the IGF-1/PI3K/Akt/mTOR pathway (Egerman and Glass, 2014), IGF-1 binds to the IGF-1 receptor which then binds with insulin receptor substrate -1 (IRS-1). Once IGF-1 has activated IRS-1, phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) is activated. (IGF-1 also activates the mitogen activated protein kinase / extracellular signal regulated kinase MAPK/ERK, but this relates more with muscle differentiation than muscle hypertrophy (Keren et al., 2006)). PI3K/Akt activation results in the activation of the mechanistic target of rapamycin (mTOR) pathway and also in the inhibition of glycogen synthase kinase β (GSK3β). mTOR is part of two complexes: the rapamycin sensitive mTOR complex 1 (mTORC1) containing raptor and the rapamycin insensitive mTOR complex 2 (mTORC2) containing rictor. mTORC2 mostly works by activating Akt. mTORC1 exerts its downstream actions by activating S6 kinase 1 (S6K1) and inhibiting eukaryotic translation initiation factor 4E- binding protein 1 (4E-BP1) leading to the activation of eukaryotic initiation factor 4E (eIF4E), a key initiator of translation (Shah et al., 2000). It is very important to note here that amino acids, and in particular leucine, are also able to independently activate S6K1 and therefore promote muscle growth (Duan et al., 2015). The inhibition of GSK3β mentioned previously also has the net effect of activating translation initiator eukaryotic initiation factor 2B (eIF2B) (Rommel et al., 2001) and promoting muscle growth.

IRS-1 is key in mediating the effects of IGF-1 (Bohni et al., 1999). Without IRS-1 the downstream effects of IGF-1 cannot happen. After IRS-1 activation, the subsequent activation of PI3K results in inactivation of IRS-1 and helps regulate the pathway (Zhande et al., 2002). Various proteins are known to 'mark' IRS-1 for degradation. Suppressor of cytokine signalling 1 and 3 (SOCS1 and SOCS3) are two of them and they are thought to mediate inflammatory related insulin resistance (Rui et al., 2002) whereas in conditions of protein scarcity, IRS-1 is degraded by F-box protein 40 (Fbxo40) (Shi et al., 2011).

Myostatin is a skeletal muscle specific member of the Transforming Growth Factor β (TGF-β) superfamily, also known as growth differentiation factor 8 (GDF 8), and is a negative regulator of muscle growth. Myostatin, together with similar acting activin-A and growth differentiation factor 11, act through the activin receptor IIB (ActRIIB). Experiments in tumor bearing mice have

shown reversal of muscle wasting by blocking ActRIIB activation (Zhou et al., 2010, Benny Klimek et al., 2010). Myostatin released into the circulation by the heart in chronic heart failure is implicated in muscle wasting (Breitbart et al., 2011). Not all studies though in humans confirm higher levels of circulating myostatin in cachexia (Loumaye et al., 2015). Binding of myostatin to its receptor leads to activation of SMAD 2 and 3 signalling mediators which translocate to the nucleus to regulate pro-atrophy gene expression (Sartori et al., 2009). Myostatin stimulation also leads to inhibition of PI3K/Akt and mTOR (Trendelenburg et al., 2009).

From the three systems of protein degradation in skeletal muscle, the ubiquitin-proteasome system (UPS) which degrades the majority of cellular proteins is thought to be the most involved in cachexia. The UPS system involves an adenosine triphosphate (ATP) dependent process of tagging protein substrates with molecules of ubiquitin. Proteins called E3 ubiquitin protein ligases are important for this 'tagging'. The polyubiquinated substrate protein is then recognised by the proteasome and degraded (Dahlmann, 2007).Two E3 ubiquitin protein ligases have been identified to be highly expressed in animal models of muscle atrophy in catabolic conditions (fasting, diabetes mellitus, renal failure and tumor implantation): muscle atrophy F box (MAFbx) (or atrogin-1) and muscle RING finger 1 (MURF1) (Lecker et al., 2004). MURF-1 has been shown to bind and ubiquitinate myosin filaments whereas MAFbx proteolyses the transcription factor eukaryotic initiation factor 3f (eIF3f). Inhibition of MAFbx activity attenuated muscle loss in fasting mice (Cong et al., 2011) as did MURF1 inhibition in cellular models (Eddins et al., 2011). Stimulation of Akt inhibits the upregulation of MAFbx and MURF 1 by the forkhead box O (FOXO) transcription factors, thus Akt has a role in inhibiting proteolysis, as well as facilitating protein synthesis (Glass, 2005).

The ubiquitin-proteasome system is the system considered responsible for mediating most of the muscle atrophy in cachexia. There is though evidence to suggest that calcium dependent proteolysis via e.g. calpains also has a role (Costelli et al., 2001), as does lysosomal protein degradation via autophagy. Even though defects in autophagy have been linked to muscular dystrophies for many years, only recently has autophagy been studied in muscle atrophy. The lysosomal-autophagy system is controlled by the FoxO transcription factors, particularly FoxO1, 3 and 4. The absence of nutrients ignites autophagy, and the formation of the autophagosome, but in FoxO 1, 3, 4 $^{-/-}$ mice this does not happen (Milan et al., 2015). In addition, Akt dephosphorylates FoxOs causing them to translocate from the nucleus to the cytoplasm, whereas when Akt is reduced, FoxOs remain in the nucleus causing upregulation of the

proteasome related atrogenes as well as autophagy related genes (Zhao et al., 2008). FoxOs are important in regulating both pathways of protein degradation.

In regards to the muscle atrophy associated with cachexia, we know that anorexia can be associated with reduced protein synthesis through reduced food intake. Nevertheless anorexia cannot explain the muscle wasting in isolation. In mouse models of cachexia there is more muscle wasting in the diseased mice than in the pair fed healthy control group (Baracos et al., 1995). Other mechanisms thought to be triggering muscle atrophy are reduced activity, potential treatment with steroids (mainly via reduced mTOR activity) and of course the presence of inflammation. Pro-inflammatory cytokines e.g. TNFα mediate muscle wasting via the activation of the transcription factor nuclear factor κβ (NFκβ) (Peterson et al., 2011), which leads to an increase in the expression of MuRF1. Pro-inflammatory cytokines also result in insulin resistance (Hirosumi et al., 2002) and therefore suppression of protein synthesis via suppressing the IGF-1/P13K/Akt pathway previously described.

The presence of muscle wasting is one of the key predictors of outcomes in chronic disease. Understanding the molecular pathways underpinning muscle wasting in cachexia is very important for the development of therapeutic targets. Potential treatments under development aiming to increase muscle mass and function include myostatin inhibitors, nutritional supplements like leucine, selective androgen receptor modulators and of course exercise. An important area of emerging research though is also the cross talk between muscle and other tissue and in particular adipose tissue.

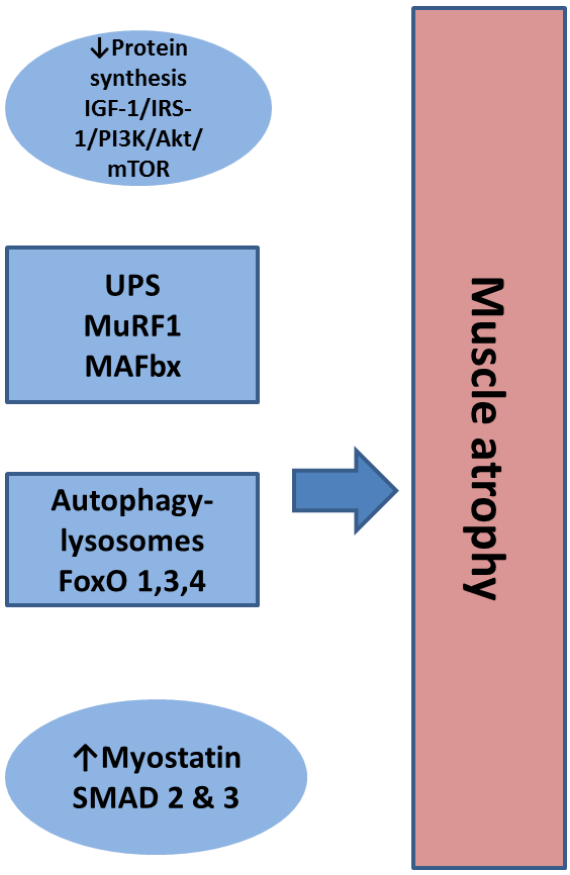

Figure 1.1 The main mechanisms of muscle atrophy in cachexia.
There are four mechanisms contributing to muscle atrophy; reduced protein synthesis, increased proteolysis by the ubiquitin proteasome system, increased autophagy and an upregulation of the negative muscle growth factor, myostatin.
IGF-1 insulin growth factor-1, IRS-1 insulin receptor substrate-1, PI3K phosphoinositide 3 kinase, Akt protein kinase B, UPS ubiquitin proteasome system, MuRF1 muscle RING finger 1, MAFbx muscle atrophy F box, FoxO 1,3,4 forkhead box 1,3,4.

1.1.4.3 Adipose tissue in cachexia

Depletion of fat mass is common in cachexia and frequently precedes the muscle loss. Adipose tissue is well known as the energy store of the body, with fat percentage ranging from 5-70% (Hausman et al., 2001). In the last couple of decades though far more interesting roles for adipose tissue in influencing energy balance are emerging. Two processes control adipose tissue mass: formation of stored fats with triacylglycerol (TAG) synthesised from dietary and endogenous non-esterified fatty acids (NEFAs), and breakdown of these stores through lipolysis, generating NEFAs and glycerol to be used as energy substrates. The main mechanism thought to be implicated in cachexia is increased lipolysis rather than decreased lipogenesis or adipocyte cell death (Ryden et al., 2008). Lipolysis is controlled by several enzymes: adipocyte triglyceride lipase (ATGL) removes the first fatty acid (FA) from triacylglycerol, and then the rate-limiting enzyme in lipolysis, hormone-sensitive lipase (HSL), removes the second. A third enzyme, monoacylglycerol (MAG) lipase removes the final fatty

acid to produce in total 3 NEFAs and 1 glycerol per triacylglycerol molecule. Lipolysis is stimulated by the sympathetic nervous system and is also influenced by hormones e.g. insulin down regulates the lipases (Blaszkiewicz and Townsend, 2016).

Inflammation is again key in generating lipolysis in the context of cachexia. Raised levels of IL-6 and its downstream products have been seen in mouse models of cancer cachexia. One study on humans showed that lipolysis was responsible for the weight loss in cancer, but that the raised levels of IL-6 in the circulation were not matched with raised IL-6 in the adipocytes (Ryden et al., 2008). TNF-α also increases lipolysis by downregulating perilipin (PLIN) (Ryden et al., 2004); PLIN is a protein that coats the fat droplets, binds to the lipolytic enzymes and prevents them from being activated. Particularly in cancer cachexia, lipolysis has also been strongly linked to the lipid mobilising factors e.g. zinc alpha-2 glycoprotein (increased in cancer cachexia) (Todorov et al., 1998).

In the more recent years research has focused on understanding the role of brown adipose tissue and also the process of 'browning' of white adipose tissue (WAT). WAT is what we mostly know as adipose tissue. WAT is known either as visceral or subcutaneous depending on its location. White adipocytes contain one large droplet of fat. BAT in adult humans is mostly found in the neck. Brown adipocytes contain multiple small fat droplets and are rich in mitochondria. They are stimulated by the SNS to generate heat as a response to cold exposure. This is done via uncoupling protein 1 (UCP1). UCP1 is located in the inner mitochondrial membrane and it can dissipate the proton gradient generated by the reactions of the citric acid cycle before it can be used to provide the energy for oxidative phosphorylation, thus releasing energy as heat. Whereas white adipocytes originate from endothelial cells, brown adipocytes have a completely different lineage and originate from mesenchymal cells that are also precursors for skeletal muscle (Sanchez-Gurmaches and Guertin, 2014). In addition, it has become apparent that some white adipocytes also share precursors similar to brown adipocytes and these are now called 'brite' adipocytes. They tend to increase in numbers when the body is exposed to cold or to β-adrenergic agonists (Sanchez-Gurmaches and Guertin, 2014). We also need to mention the presence of adipocytes in the muscle, a feature of various muscular dystrophies. In mouse models these have been shown to express high levels of UCP1 when induced by bone morphogenetic protein 7 (BMP7) (Almind et al., 2007).

Adipose tissue is no longer thought to be a passive store of energy for the body and is now considered a very active and important endocrine organ. The two most studied adipokines

produced by adipose tissue are leptin and adiponectin. Leptin reduces food intake via its receptors in the hypothalamus, increases glucose metabolism in the body whist decreasing glycogen content in the liver without an increase in insulin and it stimulates fatty acid oxidation in skeletal muscle (Harwood, 2012). Low leptin levels are associated with increased fat mass in healthy individuals, but in cachexia leptin levels are low in spite of reduced fat mass (Smiechowska et al., 2010). Adiponectin has a strong negative correlation with the amount of fat mass and low levels of adiponectin are linked to insulin resistance. There are two receptors for adiponectin, one expressed in the liver and one in skeletal muscle. Adiponectin is able to increase insulin sensitivity by reducing glucose production by the liver and by increasing fatty acid oxidation in the liver and muscle (Harwood, 2012). Paradoxically though high adiponectin levels are associated with cardiac cachexia (McAloon et al., 2016).

In summary, fat loss is an important feature of cachexia with a prognostic significance for cachexia inducing diseases. The primary mechanism involved is lipolysis, but adipose tissue also has an important role in cachexia because of its role in energy balance and because of the cross talk between adipose tissue, the brain, the skeletal muscle and the liver.

1.1.4.4 The role of the liver in cachexia

The liver is key to many metabolic processes of the body. Less is known about the role of the liver in the development of cachexia when compared to other tissues like skeletal muscle and adipose tissue. The liver is responsible for the acute phase response. This means that, when there is inflammation in the body, the liver has to shift to synthesizing acute phase proteins like CRP, serum amyloid A (SAA) etc. In fact amino acids like alanine from muscle proteolysis are diverted to the liver for gluconeogenesis and protein synthesis (Argiles et al., 1997). The liver, as previously mentioned, contributes significantly to the whole body energy expenditure and increases in resting energy expenditure have been reported in cachexia. There is experimental evidence to indicate that adenosine triphosphate (ATP) synthesis in liver mitochondria is inefficient in cancer cachexia (Tsuburaya et al., 1995, Hochwald et al., 1996) and this seems to be related to cardiolipin content of the hepatic mitochondria and is affected by stimulation by TNFα (Peyta et al., 2015, Julienne et al., 2014).

Insulin resistance has been frequently described with chronic disease and is therefore associated with cachexia, but not a defining feature of cachexia. The insulin resistance noted may be hepatic (e.g. in sarcopenic obesity) (Bhanji et al., 2017) or may be peripheral (muscles) (Cleasby et al., 2016). Most commonly in the context of cachexia it is peripheral and in cirrhosis

it has been well described that the insulin resistance is peripheral and not hepatic (Selberg et al., 1993).

In summary, as pictured in Figure 1.2, inflammation is a key process driving catabolism in cachexia. Chronic disease with chronic inflammation results in neuroinflammation and this in turn results in muscle wasting, because of accelerated proteolysis and reduced protein synthesis, fat loss, because of lipolysis and an increase in energy expenditure. One of the key metabolic pathways controlling energy balance in our cells is that of AMPK.

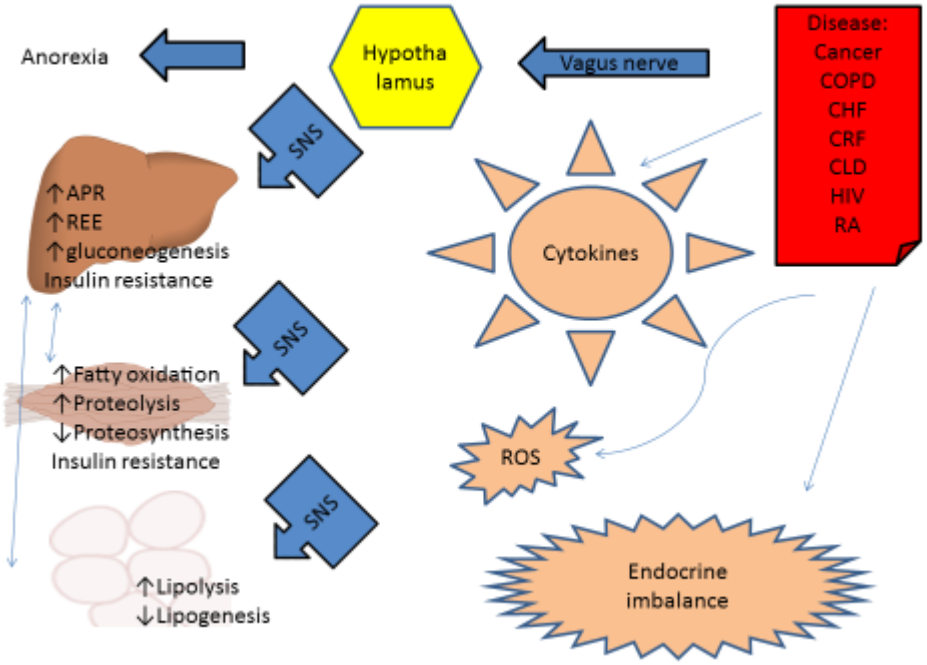

Figure 1.2 The multiple interactions leading to cachexia.
A variety of chronic diseases like cancer, COPD, CHF etc. can trigger the development of cachexia through an increase in cytokines, in ROS and by causing an endocrine imbalance. These in turn can mediate signals to the hypothalamus either directly or via the vagus nerve and directly to peripheral organs like the liver, the muscle and the adipose tissue. In addition, the hypothalamus communicates changes to the periphery via the SNS. The net result is anorexia and perturbations of metabolism consistent with catabolism.
COPD chronic obstructive pulmonary disease, CHF chronic heart failure, CRF chronic renal failure, CLD chronic liver disease, RA rheumatoid arthritis, ROS reactive oxygen species, SNS sympathetic nervous system, APR acute phase response, REE resting energy expenditure.

The pathway of AMP-activated kinase (AMPK)

AMPK is considered the master switch for the cell's energy control (Carling et al., 2011). AMPK is activated via phosphorylation when AMP levels rise in the cell indicating a shortage of ATP, the cell's energy unit. Once AMPK is activated cell proliferation stops and the cell shifts to catabolic rather than anabolic processes. AMPK, as pictured in Figure 1.3, facilitates better

glucose utilisation and lipid break down whereas discourages gluconeogenesis, lipogenesis etc. and therefore plays a crucial role in controlling cellular energy balance (Viollet et al., 2010, Steinberg and Kemp, 2009). AMPK interacts with other important pathways of substrate metabolism in the cell e.g. it inhibits phosphatidylinositol-3-kinase/ protein kinase b (PI3K/Akt) and mammalian target of rapamycin/ ribosomal protein S6 kinase beta-1 (mTOR/S6K1) (Aguilar et al., 2007, Yang et al., 2010b) in an effort to stop protein synthesis and reduce energy expenditure. AMPK also integrates stress responses, nutrient and hormonal signals to control food intake, energy expenditure and substrate utilisation at whole body level (Viollet et al., 2010, Steinberg and Kemp, 2009, Woods et al., 2005). Apart from effects in the short term, AMPK also exerts control in the long term by regulating gene transcription (Viollet et al., 2006).

AMPK is a serine/threonine protein kinase. It is a heterotrimeric complex consisting of a catalytic subunit α and two regulatory subunits β and γ. Each subunit has two or three isoforms encoded by different genes (α1, α2, β1, β2, γ1, γ2, γ3) and as a result a large variety of heterotrimeric complexes (Viollet et al., 2006, Viollet et al., 2010, Steinberg and Kemp, 2009) exist. The AMPK complexes found in the liver contain α1 and α2 subunits equally and then mainly β1 and γ1 subunits (Viollet et al., 2006).

When the cell's unit of energy, adenosine triphosphate (ATP), is depleted, adenosine monophosphate (AMP) increases, as does the AMP: ATP ratio. This results in activation of AMPK. AMPK is allosterically activated by AMP binding to its γ subunit (Xiao et al., 2007). AMP also makes AMPK more susceptible to phosphorylation by its upstream kinases and also less susceptible to dephosphorylation (Hardie, 2004). Two upstream kinases that activate AMPK have been identified in humans, liver kinase B-1 (LKB1) (Woods et al., 2003) and calmodulin-dependent protein kinase β (Woods et al., 2005). The major AMPK kinase activity in the liver though is LKB1. If LKB1 is depleted, AMPK's sensitivity to stimuli, that normally activate it, is reduced (Steinberg and Kemp, 2009, Viollet et al., 2006, Viollet et al., 2010).

Once activated AMPK has multiple roles in the liver. Its main aim is to switch on catabolic pathways and to switch off anabolic pathways. In regards to lipid metabolism, AMPK phosphorylates acetyl-CoA carboxylase (ACC) and 3-hydroxy- 3- methyglutaryl-coenzyme A reductase (HMG-CoA reductase). These two enzymes are key respectively in fatty acid and cholesterol synthesis; both ATP consuming pathways. AMPK also increases mitochondrial density (Steinberg and Kemp, 2009).

In regards to glucose metabolism, AMPK has a role in increasing glucose tolerance, reducing blood glucose levels and inhibiting hepatic gluconeogenesis. This is done in multiple ways, e.g. by activating protein AS160 (with or without insulin dependent pathways), by activating insulin receptor substrate-1 (IRS-1), by transcriptional regulation of the transmembrane protein glucose transporter 4 (GLUT4) and the enzyme hexokinase. AMPK also activates glycogen phosphorylase and inhibits glycogen synthase, therefore favouring glycogen breakdown; the long term effect of AMPK on glycogen stores is not clear (Steinberg and Kemp, 2009, Halse et al., 2003).

AMPK also has a role in inhibiting pathways of protein synthesis and cell proliferation. AMPK can inhibit the mTOR/S6K1 pathway which drives cell growth and proliferation, but can also be inhibited by S6K1 (Aguilar et al., 2007). Plus AMPK is activated by two known tumour suppressor genes: LKB1 (associated with Peutz-Jeghers syndrome) and TSC2 (associated with tuberous sclerosis). Evidence is also emerging that AMPK is also associated with muscle atrophy (Krawiec et al., 2007).

AMPK has been extensively studied in cancer cells and in obesity and diabetes type 2 (Jeon, 2016, Weikel et al., 2016). Cancer cells have ways of suppressing AMPK activation and therefore continuing to exploit the cell's energy producing mechanisms for the benefit of their own growth and proliferation (Swinnen et al., 2005). Interest in AMPK has further expanded as it has been found that metformin and the thiazolidinediones, medications used to treat diabetes type 2, activate AMPK (Coughlan et al., 2014). AMPK is therefore being recognised as a potential target for treatment of obesity, diabetes type 2 and glucose intolerance.

The role of AMPK in end stage liver disease has not been studied per se. As previously described, patients with liver disease, when compared to healthy controls, exhibit a metabolic syndrome characterised by hypermetabolism, insulin resistance and increased lipid utilisation, as well as loss of muscle mass. AMPK is key in controlling glucose and lipid metabolism in the body particularly in the liver and muscle. One could hypothesize that AMPK is activated in catabolic patients with ESCLD. AMPK activation though promotes insulin sensitivity, so it is not clear how AMPK activation interplays with the development of insulin resistance in catabolism. Thus, establishing how AMPK and related pathways react in end stage liver disease would be a further step in understanding how disease affects organ and subsequently whole body metabolism and may help develop new therapeutic interventions.

In summary, end stage chronic liver disease in adults and in children has a significant effect on their body composition mainly with weight loss, fluid retention, and loss of fat mass, loss of lean mass and with perturbations of the energy balance. This metabolic syndrome is what is now described in the context of many other chronic diseases as cachexia. Cachexia is a catabolic process involving many different integrating pathways. One of these major catabolic pathways is the AMPK pathway. AMPK has been directly and indirectly implicated in muscle atrophy, lipolysis in cachexia and it has a role in insulin sensitivity. The role of AMPK in cachexia of end stage liver disease is still to be elucidated.

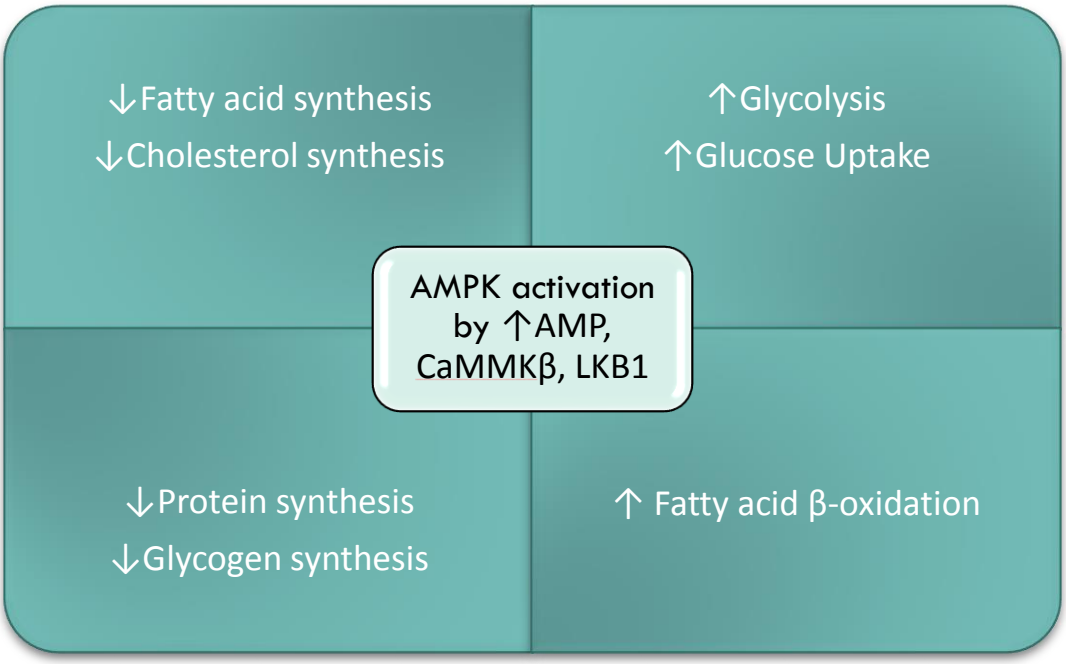

Figure 1.3 Effects of AMPK on human metabolism
AMPK AMP-activated kinase, CaMMKβ calmodulin dependent protein kinase-β, LKB1 liver kinase β-1

Hypothesis and Aims

As described in the previous section, adult patients with end stage liver disease are likely to have weight loss, fat loss and muscle loss and a proportion of them are hypermetabolic. This cachectic phenotype of weight loss, sarcopenia and hypermetabolism does not necessarily relate to disease severity. It may persist after the liver transplant and influence long term outcomes. Similar issues have been described in children, but there is often an assumption that all children of their cohort are hypermetabolic rather than a percentage of them. In addition, detailed body composition studies before and after liver transplant are lacking in children.

Body composition studies in children with liver disease are few for a number of reasons. Studying body composition in children has two main challenges; one the inherent difficulty in measuring a small child, who may find it difficult to comply with instructions and two the need for appropriate reference data per method of body composition which is not always available. In addition, as previously described, liver disease comes with changes in body composition like water retention, disproportionate abdominal distention due to organomegaly or ascites rendering many methods of body composition assessment problematic and inaccurate, particularly when used in isolation. Finally, liver disease in children is rare.

For all the above reasons, studying body composition systematically in children with end stage liver disease and their energy requirements before and after liver transplantation and attempting to add to the body of knowledge in this field becomes imperative.

1.1.5 Clinical Questions

i. What is the body composition and resting energy expenditure of children with end stage chronic liver disease pre and post liver transplant?

ii. Do children with a higher resting energy expenditure per weight have more complications and worse outcome post liver transplant and does the increase in resting energy expenditure persist 6 months post liver transplant?

iii. How are metabolic pathways, that control cell energy metabolism, affected in children with end stage chronic liver disease and how do they relate to body composition, resting energy expenditure and outcome?

1.1.6 Hypothesis

A proportion of children with end stage liver disease are hypermetabolic and cachectic. These children suffer more complications after their liver transplant. Cell energy controlling metabolic pathways are likely to be disturbed and contribute to the disease state.

1.1.7 Aims

Primary aims:

- To study the role of cell energy controlling pathways in children with end stage liver disease.
- To study how these relate with their REE, body composition and their outcome post liver transplant.

Secondary aims:

- To measure and describe body composition and resting energy expenditure in children with end stage liver disease and how they influence outcome post liver transplant.
- To study how body composition and resting energy expenditure change at least 6 months post liver transplant.

Chapter 2 MATERIALS AND METHODS

Research Protocol

Children were prospectively enrolled as they came for their liver transplant assessment. This meant that they had end stage liver disease including one or all of the following: liver synthetic failure, development of portal hypertension, persistent jaundice, growth failure resistant to nutritional management, refractory episodes of cholangitis/ bacterial peritonitis and poor quality of life.

The study was comprised of **two arms**.

The **first arm** involved detailed assessment of body composition and resting energy expenditure of the patients before and after liver transplant. The first arm also involved recruiting healthy participants that were age and sex matched to the patients.

Inclusion criteria:

Children with end stage liver disease coming for their liver transplant assessment.

Age 6 months-18 years

Consent to participate

Exclusion criteria:

Children with acute liver failure (children without a previously known liver disease presenting with liver failure of a relatively short history and requiring a liver transplant)

Children with liver tumours e.g. hepatoblastoma, without evidence of chronic liver disease

Healthy controls were recruited from siblings of patients with liver disease or other healthy children. The aim was to recruit healthy children that were age and sex matched to the patients so that the two groups could be comparable. Siblings were matched to other patients in the study for which they were an age and sex match.

Pre liver transplantation body composition assessment for the patient participants

(Figure 2.1)

Clinical review and examination

Liver ultrasound (performed by the radiology department as part of their clinical review)

Weight and height measurement (details in section 2.1.1 page 46).

BMI calculation

Mid upper arm circumference

Multiple skin fold thicknesses

Multifrequency bioelectrical impedance for all ages (details in section 2.1.6 page 54).

Indirect calorimetry to measure resting energy expenditure (details in section 2.1.5 page 52).

For the over 4 years of age: 4 component model of body composition.

Air displacement plethysmography (BOD POD) to measure body volume (details in section 2.1.2 page 47).

Whole body DXA scan to determine bone mineral content (BMC) (details in section 2.1.3 page 48).

Bromide dilution studies to measure extra cellular water (ECW) (details in section 2.1.4 page 50).

3D photonic scanning to assess body shape (details in section 2.1.7 page 57).

For the under 4 years of age: 2 component model of body composition.

Deuterium dilution studies (details in section 2.1.4 page 50).

Bromide dilution studies (details in section 2.1.4 page 50).

The under 4 year olds could not have a 4 component model, because of the technical difficulties and lack of reference data when using the BOD POD and the DXA scan in this age group.

Blood sampling included full blood count (FBC), urea and electrolytes (U+Es), liver function tests (LFTs), albumin, international normalised ratio (INR), vitamin A, E and D levels, lipid profile, thyroid function tests, cortisol and baseline samples for the dilution studies. Two more blood tests were required for the dilution studies and these were taken from an intravenous cannula inserted for the first blood test.

Body composition assessment post liver transplant.

This assessment was identical to the initial assessment and was undertaken at 6 months post liver transplant, for the patients that were well enough, or anytime thereafter.

Body composition for the healthy participants:

The healthy participants had one assessment which included anthropometry, indirect calorimetry, BOD POD (if they were over 4 years of age), deuterium dilution studies and multifrequency bioelectrical impedance. They did not have a DXA scan and bromide dilution studies. No blood samples were taken from the healthy participants, urine samples were obtained for the deuterium dilution studies.

Other data collection:

During the whole study period for each patient i.e. from the time of initial assessment till the post liver transplant assessment the following were recorded:

Length of stay in hospital (PICU/ HDU/ total)

Infection episodes (bacterial and viral)

Graft function (episodes of acute cellular rejection, biliary complications, vascular complications, liver function tests)

Final outcome (alive, re-transplanted, death).

All the anthropometrics were obtained by the same operator.

For the second arm of the study:

Inclusion criteria:

Children undergoing a liver transplant with end stage liver disease with consent to participate (the patients from the first arm of the study) and children undergoing a liver transplant without end stage liver disease e.g. with Crigler-Najjar type 1 syndrome.

Age from 6 months-18years

Consent to participate

Exclusion criteria:

Children with acute liver failure or tumours undergoing a liver transplant (as previously stated).

Liver tissue from the explanted liver, muscle tissue (e.g. from the rectus abdominis) and adipose tissue (e.g. from the subcutaneous fat) was collected during the liver transplant operation to be used for assessment of cell energy controlling metabolic pathways. In particular, levels of expression and activation of precursor activators of the AMPK pathway, like LKB-1 were studied, as well as relevant subunits of AMPK and of course downstream to AMPK enzymes, like ACC1, ACC2. Pathways interacting with the AMPK pathway with a particular interest in pathways involved in muscle wasting and lipolysis were also studied.

Tissue from the children without end stage liver disease undergoing liver transplant were used as comparison/controls to the ones with end stage liver disease. We aimed to recruit 1-5 of these.

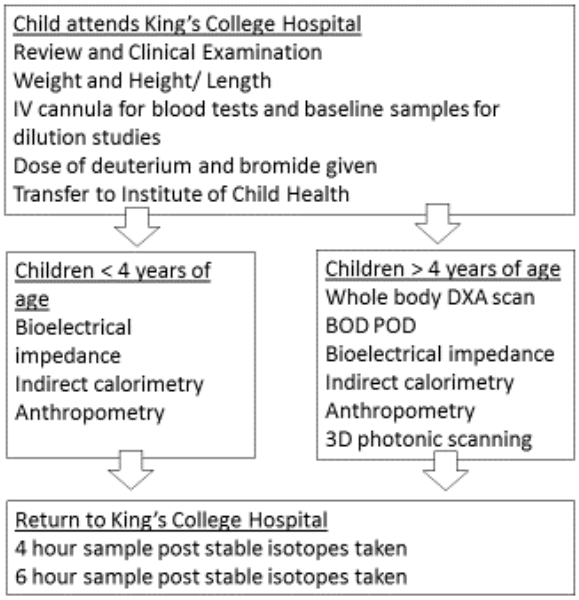

Figure 2.1: Pathway for the body composition assessment of the patient participants
The children would start the body composition assessment at King's College Hospital where they also they had their clinical review. After the stable isotopes were given, the assessment would continue at the Institute of Child Health before the final samples for the dilution studies were taken back at King's College Hospital.
DXA dual-energy X-ray absorptiometry, BOD POD air displacement plethysmography

Ethical Approval and Amendments

Ethical approval was obtained from the NRES Committee London – Central (11/LO/1146). Two amendments to the protocol have also received ethical approval. The first one was in regards to the number of samples required for the stable isotope studies (from two samples the protocol was changed to three). This change was necessary, because we changed provider for the stable isotope studies. The second one was in regards to using plasma instead of saliva samples for the deuterium measurements for the patients and urine samples instead of saliva samples for the controls. This change was necessary because the smaller children could not provide adequate saliva samples.

Methods of Body Composition

There is no gold standard of measuring body composition *in vivo* in humans. Various methods of body composition have been developed that can be used in isolation or in combination as explained later on. Each method has its own advantages and disadvantages. When measuring children, one needs to keep in mind that the less cooperation needed from the child the more accurate the measurements, In addition, because children grow, there need to be normative to compare their measurements. If a method can also be applied by the bedside, then it could be

more helpful for sick children. The following table summarises the advantages and disadvantages of the methods used in this study.

Table 2-1: Advantages and disadvantages of body composition methods used

Method	What it measures	Advantages	Disadvantages
Anthropometry	Weight, Height, skinfold thickness	Simple Bedside Normative data exist	Variation between operators
Air displacement plethysmography	Body volume	Safe	High degree of cooperation required Normative data only for over 5 year olds and under 6 month olds
Whole body DXA	Bone mineral density	Easy method Children enjoy the result	Low dose radiation Normative data only for over 5 years of age
Deuterium dilution studies	Total body water	Established method Normative data exist	Method has many steps- high degree of precision required Child's cooperation required Requires saliva, urine or blood sampling
Bromide dilution studies	Extracellular body water	Established method	Method has many steps- high degree of precision required Child's cooperation required Requires blood sampling
Indirect calorimetry	Energy expenditure	Simple Safe	Requires a fasting period A degree of cooperation required
Bioelectrical impedance	Resistance and reactance	Quick Simple Safe	Lack of normative data
3D photonic scanning	Body shape	Children enjoy this scan	A degree of cooperation required Not an established method of body composition

DXA dual X-ray absorptiometry, 3D 3 dimensional

As there is no gold standard of measuring body composition *in vivo* in humans, various models exist that attempt to estimate the various body compartments based on a combination of measurements and on assumptions. The more assumptions one makes the less potentially accurate the estimations.

The 2 component (2C) model (Wells and Fewtrell, 2006, Wells et al., 1999) divides body mass into fat mass (FM) and fat free mass (FFM). Body weight (BW) is the sum of FM and FFM.

BW= FM + FFM

Once we measure BW, by estimating FM we can estimate FFM or the other way round. FM can be estimated using various equations, one very well- known one being Siri's equation where BD is body density (Siri, 1993):

FM% = [(4.95/BD) – 4.5] * 100

The body density (BD) in Siri's equation can be estimated either by measuring skin fold thickness or by measuring body volume (BV).

With skin fold thickness (4 measurements: subscapular, biceps, triceps and suprailiac) there are various equations that can be used. For example, Johnston's equations for children (Johnston et al., 1988):

For boys: **BD= 1.166- 0.070 x**, where x is the log of the sum of the 4 skinfold measurements

For girls: **BD= 1.144- 0.060 x**.

FM in children can also be estimated using the equations of Slaughter et al that require just triceps and subscapular skinfold measurements (Slaughter et al., 1988).

BV can be measured by hydrodensitometry or by air displacement plethysmography (BOD POD) and BD= BW/BV (kg/litres). In addition, if one has BD from BV, they can use the modified Siri's equation (Siri, 1956):

F_f= [(D_{FFM}/BD)-1]/ [(D_{FFM}/0.9007)-1]

In this equation F_f is the fraction of body weight that is fat mass, D_{FFM} is the density of the fat free mass, which particularly in children is not constant and 0.9007 is the density of fat which is constant. D_{FFM} can be estimated for children of various ages from the equations of Weststrate and Deurenberg (Weststrate and Deurenberg, 1989) which require just age to be solved.

Alternatively we can estimate the FFM by measuring TBW and by assuming hydration of FFM is constant. For adults this is assumed to be 73% (Wang et al., 1999) i.e. TBW= 73% FFM. In children, we know hydration is not constant. Two hallmark papers can be used when converting TBW estimates to FFM, one is that of Lohman (Hewitt et al., 1993) that provides hydration factors for male and female children from their first year of life till the age of 16 years and the other is that of Wells et al (Wells et al., 2010b). In this more recent paper, hydration factors are provided for children of both sexes from the age of 4 years to 23 years, based on body composition analysis of 533 healthy children.

With the 3 component (3C) model (Wells and Fewtrell, 2006, Wells et al., 1999) the body is divided into fat, water and fat free dry mass; assuming that the protein: mineral ratio is constant.

By measuring body volume (BV) in litres, total body water (TBW) in litres and body weight (BW) in kg, we can estimate fat mass.

FM kg= [(2.22 * BV)- (0.764 * TBW)] – (1.465 * BW)

With the 4 component model (Wells and Fewtrell, 2006, Wells et al., 1999) the body is divided into fat, water, protein and mineral. This model does not assume that the ratio of protein to mineral in fat free mass is constant. The ratio of bone mineral to total body mineral is still assumed to be constant.

FM (kg)= [(2.747 * BV) – (0.710 * TBW)] + [(1.460 * A) – (2.050 * BW)]

In this equation: BV is body volume from displacement plethysmography in litres, TBW is total body water in litres, A is total body mineral content (BMC) from the DXA scan in kg and BW is body weight in kg.

This study uses the 2C, 3C and 4C models for the children over 4 years of age and the 2C model for the children under the age of 4 years. The reason for the difference is that the techniques available to us for the 4C model are not all applicable to younger children either from a practical point of view e.g. a 2 year old cannot stay still for the air displacement plethysmography for the required amount of time or because of lack of reference data e.g. with DXA. In addition, because in chronic liver disease it is well known that there is an increase in extracellular water (ECW), we are estimating TBW and ECW. In a study comparing a 4C model of body composition of 20 cirrhotic patients without overt oedema to 20 healthy individuals, Morgan et al were able to show that the density of the FFM in the patients was in fact lower and with a higher hydration factor (Morgan et al., 2006) than in the controls, indicating that 2C models are not accurate to use on patients with cirrhosis. On the other hand being able to compare 4C with 2C models can give us some insight as to which 2C method would be the most appropriate to use in our daily practice where of course the 4C cannot be used.

2.1.1 Anthropometry

Weight was measured for the over 4 year olds in swim wear to the nearest 0.01 kg. The measurement was repeated and recorded twice after excluding any erroneous values. The younger children were weighed with no clothes. Height was measured on a wall mounted stadiometer to the nearest 0.1 cm for the over 4 year olds. The measurement was repeated and recorded twice after excluding any erroneous values. For the younger children length was measured on a horizontal stadiometer. BMI was calculated as weight/ height^2, with weight in kilograms and height in metres. The raw weight, height and BMI measurements were converted

to sd scores (standard deviation scores otherwise known as z scores) using the 1990 reference data (Cole et al., 1995).

Mid-upper arm circumference (MUAC) was measured according to the Institute of Child Health standard operating procedure with appropriate tape and on the left arm. The circumference was measured at the half point between the acromion tip and the point of the elbow. The half point was identified with the left arm at the side and the elbow bent at 90º whereas the circumference was measured with the arm hanging loosely by the side of the body. The measurement was recorded to the nearest 0.1 cm and was repeated three times.

Skinfold thickness was measured for biceps, triceps and subscapular skinfolds on the left side with Holtain callipers according to the Institute of Child Health standard operating procedure. For the biceps skinfold the point of measurement was on the anterior aspect of the arm, on a line perpendicular to the line drawn for the MUAC and directly over the humerus. For the triceps it was on the posterior aspect of the arm and on a line perpendicular to the line drawn for MUAC and directly over the humerus. The subscapular skinfold was measured just below the inferior angle at the lower margin of the scapula with the skinfold inclined 45º to the horizontal and in the natural cleavage line of the skin. Measurements were repeated and recorded 3 times and to the nearest 0.2 cm after excluding any erroneous values.

All patient participants and all healthy controls had their anthropometry done.

2.1.2 Air displacement plethysmography (BOD POD)

Body volume (BV) is required for the 3C model (done for the healthy controls over 4 years of age) and for the 4C model (done for the patients over the age of 4 years). As we previously discussed, body volume BV can also be used together with weight in estimating fat and fat free mass as a 2C model. BV can be measured by hydrodensitometry (a technique also known as hydrostatic weighing or underwater densitometry) or by air displacement plethysmography using the BOD POD (Model Siri 1961, Life Measurement Instruments, Concord, CA). The latter method was developed as an easier quicker, more appealing method, particularly as it does not require submersion in water. Indeed it has been shown in children that percentage of body fat (%BF) estimated by BOD POD has a high correlation with %BF estimated by DXA and by hydrodensitometry (Lockner et al., 2000, Nunez et al., 1999).

This technique is used for the children over 4 years of age as it requires a certain degree of co-operation from them. The machine has an anterior and a posterior chamber with an oscillating diaphragm between the two chambers creating volume perturbations that lead to small pressure

fluctuations. The machine operates on the basis of Boyle's Law which states that at a constant temperature (isothermal conditions), the product of pressure (P) and volume (V) is constant i.e. P1V1= P2V2. It makes measurements of volume by calculating the difference in volume in the anterior chamber with and without the patient sitting in it.

The participants have their measurements taken wearing a tight fitting swimming costume and a swimming cap. Two complete tests are performed on each child; these provide at least 4 raw volumes of total body volume (in litres). The raw body volume needs to then be converted to actual body volume. This is done by correcting for thoracic gas volume and the surface area artefact by using appropriate equations for children that take into account their age, sex, weight and height (Dewit et al., 2000). Lung volume was therefore predicted using age and sex specific equations (Wells and Fuller, 2001), as actually measuring lung volume particularly in young children is quite challenging.

Figure 2.2: The BOD POD

2.1.3 Whole body dual-energy X-ray absorptiometry (DXA)

DXA was originally designed to measure bone mineral content (BMC) expressed in grams (g) and bone mineral density (BMD) expressed in grams per square centimetre (g/cm^2), but it has also been applied for body composition assessment. DXA machines use a source of X-rays at two energies, low and high energy, a detector and a computer system that converts the data into an image. Depending on the path of the X-ray beam DXA machines can be pencil beam, fan beam or narrow fan beam. The first scanners were pencil beam. Subsequently the fan beam ones allowed for better resolution and faster scanning times at the cost of higher radiation doses and magnification of scanned areas closest to the source of X-ray. The narrow fan beam

scanners like the Lunar Prodigy used in this study use a wider beam than the pencil beam, but narrower than the fan beams and therefore are still quick with a reduced magnification effect (Laskey, 1996, Toombs et al., 2012).

DXA assumes the body is of two components; bone mineral and soft tissue. As the X-ray beam travels through the body its two different energies are attenuated. By using the two energies of the un-attenuated and the attenuated beam the computer is able to conclude if the pixel can be assumed to contain bone or soft tissue. In an average subject about one third of pixels are over bone and 2/3 are over soft tissue (Toombs et al., 2012, Laskey, 1996).

DXA is used in the 4 component model to measure body mineral content (Wells and Fewtrell, 2006). DXA is also used in studies as a stand-alone tool for body composition. The advantages of DXA are that it is a very easy to perform technique as long as the subject can lie still on their back for a few minutes, this may of course be a challenge for young children, and that it can provide regional body composition measurements, which are very important in individuals with altered distribution of body fat and loss of lean mass. The disadvantages of DXA are that it involves low radiation doses (estimated radiation exposure per scan with the Lunar Prodigy 2.2 μSv) and that it assumes a constant hydration factor for the fat free mass of 73%. This means that in individuals with a higher hydration factor, as may be the case in patients with liver disease, DXA will overestimate fat mass (Laskey, 1996). In addition hydration of lean mass in children varies with age and sex (Wells et al., 1999). DXA measurements have not been compared to cadaveric body composition data and not all DXA systems have been compared to gold standard in vivo methods like the 4 component model (Toombs et al., 2012). Furthermore differences have been consistently noted in measurements between different DXA systems (Toombs et al., 2012, Laskey, 1996). Where DXA has been compared to the 4 component model it has been mostly found to underestimate fat mass (Toombs et al., 2012). A study though comparing the Lunar Prodigy scanner, used in this study, with the 4 component model in healthy individuals showed that it overestimated fat mass (Williams et al., 2006). The same group has compared DXA body composition data to the 4 component model in anorexic and obese young people compared to healthy controls and has found that in both cases DXA overestimated fat mass and underestimated fat free mass (Wells et al., 2010a, Wells et al., 2015a).

Therefore DXA has, in spite of significant advantages, significant limitations when applied to assess body composition in individuals expected to have altered hydration of fat free mass

and/or excessive loss of fat free mass. As previously mentioned, in this study, the Lunar Prodigy Advance PA+ 303999 whole body scanner was used (GE Medical Systems, Madison, WI, with software Encore 2002). For the DXA scan the patients wear loose fitting clothes and lie down on their back for about 5-10 minutes. Only patients that were over 4 years of age had the DXA, as part of the 4C model. From the DXA scan we were able to obtain values for bone mineral content, fat mass, and bone free fat free mass both regional and for the whole body.

Figure 2.3: The DXA scanner

2.1.4 Deuterium and bromide stable isotope dilution studies.

Stable isotopes are atoms whose nuclei contain the same number of protons, but different numbers of neutrons. Isotopes occupy the same position on the periodic table. Isotopes can be radioactive or stable. Radioactive isotopes can spontaneously disintegrate to form an atom of another element sometimes omitting radiation in the process, whereas stable isotopes have stable nuclei and pose no physiological risk. For example deuterium (^2H) is the stable isotope of hydrogen. Hydrogen has one proton in its nucleus whereas deuterium has one proton and one neutron.

Deuterium dilution studies are used for the estimation of TBW (Schloerb et al., 1950). TBW measurements can be used as part of the 2C model and are also needed for the 3C and 4C models. Bromide dilution studies are used for the estimation of the ECW. TBW is ECW plus intracellular water (ICW). ECW includes interstitial fluid, plasma volume and fluid compartments like cerebrospinal fluid and joint fluid. The basic principle of dilution studies is that we give a certain dose of a tracer and after it has reached the equilibrium phase of its distribution in the fluid compartment of interest, we measure its concentration in that compartment. The volume of

the fluid compartment of interest will equal the mass given (the dose) divided by the concentration of the tracer (Dou et al., 2012).

Deuterium labelled water (D_2O) is handled by the body just like water. The dose of deuterium used in children is 0.05 gr/kg BW of patient (99.8% D_2O, Cambridge Isotope Laboratories) (Chomtho et al., 2006). Our patient participants had their deuterium measured with the equilibrium (or plateau) method, whereas the healthy participants had the deuterium measured by the back extrapolation method. After the dose administration one has to wait for the dose to equilibrate with the total body water pool. For deuterium this is 4 hours. The dilution space (N) is calculated from the following equation:

$$\mathbf{N = TA/a * (E_a - E_T)/ (E_s - E_p)}$$

where A is the isotope dose in grams, a is the isotope dose in grams retained by the machine for the analysis, T is the amount of tap water in which a is diluted in before analysis and E_a, E_T, E_s, E_p are the isotopic enrichments in delta units in the dose retained by the machine, in the tap water, in the sample of plasma post equilibrium and in the sample of plasma before the dose is given (Wells et al., 2005). Delta units express isotopic enrichment relative to a standard, in this case Vienna-Standard Mean Ocean Water.

For the equilibrium method, first a blood sample was obtained from the patient and then the dose was administered. The administering vessel (cup or bottle) was first weighed empty, then with 10 mls of water and then with the deuterium. The individual weights and volumes were recorded. A 1 ml sample of the solution was removed and the vessel was weighed again. If a straw was added, an additional weight with the straw was obtained. The solution was then given to the participant to drink. The time of drink was recorded and the vessel was weighed again after the participant has finished drinking. At 4 hours and 6 hours after the drinking time, blood samples were obtained and the exact time of the sampling was recorded. The plasma from the blood samples was stored in -80°C and was analysed in batches at the Cambridge MRC- HNR centre by Dr L Bluck's team with IR-MS (isotope ratio mass spectrometry).

For the back extrapolation method, a urine sample was collected from the child prior to giving the dose of deuterium. The dose was given just like the previous method and the same times and measurements were recorded. A further 5 samples of urine were collected by the parents starting the following day and for 5 days and the date and time of the urine collection was recorded. For the infants that were still in nappies any urine sample at any time was acceptable, but the continent children had to give us urine samples after the first one of the day.

E_s with this method was calculated from the washout kinetics of the isotope as indicated from these 5 samples (Wells and Strickland, 1996). The equilibrium method overestimates TBW in infants under the age of 2 years, but in children over the age of 2 years the two methods yield similar results (Wells et al., 2005). The patients under the age of 2 years had the equilibrium, because the sampling could be combined with the samples for the bromide dilution.

With the bromide dilution method the corrected bromide space (CBS) is measured and we are assuming that after some adjustments this equals ECW. Bromide has good oral absorption, tends to not penetrate in cells and has a slow excretion, therefore the bromide space represents the extracellular fluid well (Sergi et al., 2003).

CBS= [Br dose in mg/ Br dose in plasma in mg/L] * 0.9 * 0.95 * 0.94

Where 0.9 is the correction for the amount thought to be distributed in non-extracellular sites, like the erythrocytes, 0.95 is the Donnan equilibrium factor and 0.94 is the concentration of water in serum (Bell et al., 1984).

The dose of bromide used was 25 mg Br/kg BW of patient (3.22% NaBr, Tayside Pharmaceuticals) (Prelack et al., 2005). This was given to the patient to drink after the pre-dosing baseline blood sample had been taken. The time the dose was taken was recorded. The administering vessel (cup) was first weighed empty and then with the bromide solution. A 1 ml sample of the solution was then taken out and the cup was weighed again. All weights and the volume of the bromide solution were recorded. The patient drank the solution, the exact time was recorded and the vessel was weighed again. Blood samples were taken at 4 and 6 hours after drinking the solution. The exact time of the sampling was recorded. The samples were centrifuged and plasma from the pre-dose sample as well as the 4 and 6 hour post dose blood samples were stored at -80ºC together with the sample of the solution. These were later analysed in batches at the Department of Biomineral Research at the MRC- Human Nutrition Research, Elsie Widdowson Laboratory. The method used for the analysis is ICP-MS (inductively coupled plasma mass spectrometry) and with this the average of ^{78}Br and ^{81}Br concentration in mg/L was measured for each sample.

2.1.5 Indirect Calorimetry

REE was quantified by indirect calorimetry using the Deltatrac II metabolic monitor (Datex-Ohmeda). The Deltatrac II is considered the most accurate open- system indirect calorimeter and has been evaluated in many studies (Wells and Fuller, 1998). It requires a 30 minute warm-up and calibration of the gas analysers using a known standard gas mixture of 95% O2and 5%

CO2. The participants were measured at least 2 hours after any oral/ enteral intake. They were asked to lie down and the mixed expired gas was collected via a canopy hood for up to 30 minutes. All our participants were spontaneously breathing. All the patients and all the healthy controls had indirect calorimetry.

The machine measures the O2 and CO2 gas fractions via gas sensors and then transforms these values to VO2 and VCO2. It was calibrated before each use. The machine requires to measure the patient in a respiratory and metabolic steady state; where the equilibrium of variation of VO2 and VCO2 must be less than <10% for five consecutive minutes (McClave et al., 2003b) or alternatively one can take measurements over 30 minutes and then discard the measurements of the first 10 minutes (Matarese, 1997, Weissman et al., 1986).

The calorimeter then uses the Weir equation (Weir, 1949) to transform VO2 and VCO2 into energy expenditure per minute with a correction for the metabolism of protein.

M = (3.941* VO2) + (1.106* VCO2) - (2.17 *uN2)

M is the metabolic energy expenditure in kcal/min and uN2 is urinary nitrogen in g per day. The equation applies to the fasting state and the error of omitting the effect of protein metabolism is small (1% for each 12.3% of total calories arising from protein metabolism) (Wells, 1998).

M (kcal/min) * 1440 min/day = REE (kcal/day).

The Weir equation is based on knowing the primary caloric value for the metabolism of a mixture of 1 g of carbohydrate, lipid and protein with the consumption of x litres of O2 (VO2) and the production of y litres of CO2 (VCO2). In addition the values of VO2 and VCO2 can be used to derive equations for determining the endogenous metabolic rate of carbohydrate, lipid and protein.

The respiratory quotient RQ is the ratio of VCO2 and VO2. RQ varies from 0.67 (ketone body metabolism in the fasting state) to 1.3 reflecting lipogenesis from glucose or hyperventilation (Holdy, 2004). Below 0.67 it suggests there may be a leak in the gas collecting system, but investigators have reported RQ below 0.67 in patients with liver disease (Glass et al., 2013). The current opinion is that using the RQ to guide the macronutrient choice of nutritional support is inadequate (McClave et al., 2003a).

In bed ridden patients REE is comparable to total energy expenditure (TEE), but in active patients TEE is made up of REE plus physical activity energy expenditure (PAEE) plus thermogenesis. PAEE is the component of TEE with the greatest variability, but has not been

evaluated in most disease states. TEE can be evaluated with double-labelled water (Levine, 2005).

Figure 2.4: Indirect calorimetry

2.1.6 Bioelectrical Impedance (BIA)

All our patient and healthy control participants had BIA. BIA is a way of estimating body composition in humans. The technique involves passing an electrical current between 2 points of the subject's body; usually these are the wrist and the ankle on the same side. It relies on the electrical properties of human tissue. It aims to measure the impedance (Z) of the current as it passes through the body. Electrical impedance consists of 2 components in human tissue; resistance (R) and reactance (Xc).

When a current passes through a cylinder of homogeneous conductive material the resistance (R) is the opposition offered by the material to its flow. This is proportional to the cylinder's length (L) and inversely proportional to its area (A) (Figure 2.5). The human body of course is not a cylinder and is not made of homogeneous material, nevertheless a relationship can be established between the impedance quotient (L^2/R) and the body water, which contains the electrolytes that conduct the current. Instead of using the length between the 2 electrodes, height of the individual can be used. As lean mass contains the water (e.g. 73% in adults), the relationship becomes between lean mass and $height^2/R$ (Kyle et al., 2004).

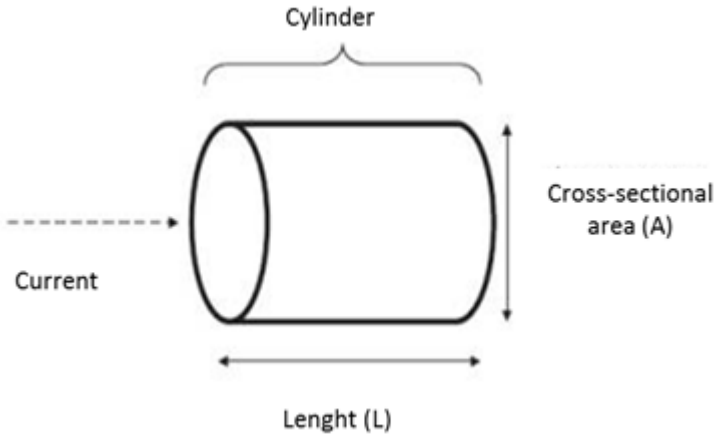

Figure 2.5: Principles of BIA. Adapted from (Kyle et al., 2004)

Reactance is related to the capacitance (i.e. the ability of a system to store electric charge) and variations in capacitance can occur depending on the integrity, function and composition of the cell membrane. At zero frequency the current cannot penetrate the cell membrane, so it passes through the extra cellular water (ECW) and the resistance it meets is R0. At infinite frequency, the cell membrane is a perfect capacitor and so the current passes through intra and extra cellular water and the resistance it meets is R∞.

Phase angle (PA) is derived from resistance and reactance. It is the arc tangent of the ratio of reactance/ resistance converted to degrees.

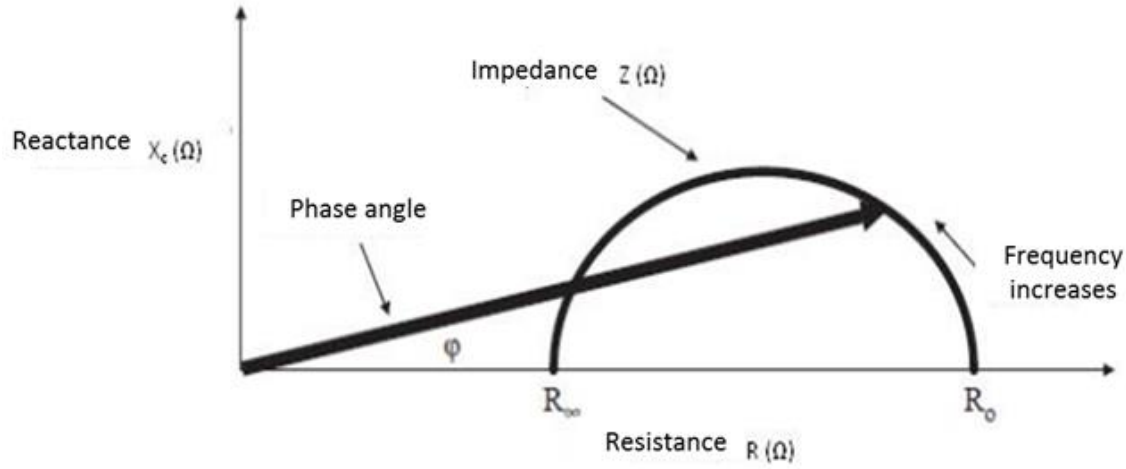

Figure 2.6: Representation of phase angle.
Adapted from (Kyle et al., 2004)

The relationship between reactance and resistance reflects different properties of tissues that are affected by disease, nutritional status and hydration. Phase angle has been interpreted as an indicator of cell membrane integrity and water distribution between the intra and extra cellular space. Another index of this type is the ratio R0/R∞. Total body phase angle has been

shown to be mostly determined by arm and leg phase angle (Selberg and Selberg, 2002). This is of particular relevance for patients with liver disease. Phase angle has been shown to correlate with total body potassium (body cell mass) and with muscle mass (Selberg and Selberg, 2002).

The illness marker (IM) or impedance ratio is the ratio of impedance at 200 kHz and 5 kHz. Impedance at 200 kHz reflects TBW and at 5 kHz ECW. The greater the variance between these 2 impedance values the healthier the cells. IM closer to 1 indicates poor cellular health or fluid overload.

BIA measures properties of fat free mass and therefore should not be used to predict fat mass. Hydration of fat free mass varies between populations depending on age and other parameters. Equations exist to convert the impedance measurements to clinically relevant data, but one needs to be aware of the characteristics of these populations when applying the equations to different individuals (Wells and Fewtrell, 2006).

BIA can be single frequency, usually 50 kHz or multi-frequency, deriving measurements at a range of frequencies usually from 5 kHz to 200 kHz. Multifrequency BIA is usually better at detecting changes in ECW (Patel et al., 1996). We performed BIA with a multi-frequency bioelectrical impedance meter (Quadscan4000). Two electrodes were placed over the dorsal aspect of the left hand and foot of the child whilst it was lying down. The readings took about 20 seconds and we did 2 measurements for each participant.

Bioelectrical impedance vector analysis (BIVA) is an attractive technique that may be used without the need for equations. The patient's R and Xc standardised for height are plotted in the R-Xc plane. An individual vector can then be compared with the 50%, 75% and 95% tolerance ellipses calculated in the same population of the same gender and race. Clinical validation studies have shown that patients falling outside the 75% tolerance ellipse have abnormal tissue impedance. These abnormalities may be related to hydration, either dehydration or over hydration or they may be related to body cell mass content (visceral/ muscle protein and intracellular water) in the lean mass (Kyle et al., 2004, Walter-Kroker et al., 2011). This a particularly exciting application of BIA, because it does not rely on equations for the data to be interpreted and it can be of direct benefit to the patient. For e.g. one can adjust the dose of diuretics so that the patient is not dehydrated or over-hydrated and one can also compare consecutive measurements of the same person and can picture how their body composition changes (Piccoli et al., 2014).

2.1.7 3D Photonic scanning (3D-PS)

3D-PS is a technique that has been used for many years in the clothing industry and has only relatively recently been used to study body composition and in particular to study body shape, body volume and to obtain various diameters, girths and volumes of the body and its segments that may be of interest.

Only the patients over 5 years of age had the 3D-PS. They had the scan in their underwear, which had to be tight fitting and light in colour, to provide contrast against the scanning booth walls which are black. The scanner projects strips of white light onto the body and records the distortion induced by the body's body surface. The scanner uses 6 fixed cameras and records over a period of about 8 seconds. Subsequently it takes this raw photonic data and reconstructs the skin topography using computer algorithms. We used the NX16 scanner ($[TC]^2$, Cary, North Carolina) and the software used is Body Measurement System version 5.3. 3D-PS measurements have shown high correlation with manual measurements, but the 3D-PS measurements have consistently been shown to be slightly but systematically higher in both adults and children (Wells et al., 2015b)

The advantage of 3D-PS is that it is a safe and quick method with which one can obtain multiple measurements and can also track or project changes in body shape. It is though (Wells et al., 2015b) a technique that is still under evaluation in regards to its application in body composition sciences.

Other data collection

For each patient we recorded the date of liver transplantation, the amount of days they were intubated after the transplant in PICU, the total of days they were in PICU/HDU and the days to discharge. We also recorded all major complications (infections, acute cellular rejection, surgical complications, post-transplant lymphoproliferative disease (PTLD)) and other reasons for re-admission to hospital.

Tissue Studies- Gene Expression Analysis

Liver tissue was collected at the time of transplant by wedge biopsy. Samples were immediately frozen in liquid nitrogen and stored at -80ºC. At the same time of transplant samples of muscle

and fat tissue were taken from the abdominis rectus and its subcutaneous fat respectively. These samples were stored in the same way at -80ºC.

The aim was to process the samples and look at gene expression by microarray analysis. For this to happen, total RNA needed to be extracted from the tissue and converted to amplified cDNA (complementary DNA) with was hybridized onto the microarray chips. Following this we performed relative quantification real time polymerase chain reaction (qRT-PCR) in order to validate the microarray findings.

2.1.8 Isolation of Total RNA

Total RNA was extracted from the tissue samples using the MagMAX™-96 for Microarrays Total RNA Isolation Kit by Thermofisher Scientific. In particular the spin procedure was used, which is suitable for tissue samples that have been snap frozen and are potentially high in fat content and/ or high in cellular content. It has fewer steps than the no-spin procedure and more tissue can be processed per sample. The procedure protocol can be found on the company's website (https://tools.thermofisher.com/content/sfs/manuals/cms_055599.pdf).

Procedure Overview:

1. Sample homogenized in TRI Reagent (1 mL TRI Reagent per 50–100 mg tissue)

-the homogenate was incubated at room temperature for 5 minutes

2. Spin Procedure
    a. Separation of aqueous and organic phase

- Homogenate mixed with 0.1 volumes BCP and left at room temp for 5 min
- Centrifuged at 12,000 x g for 10 min at 4°C
- 100 µL of aqueous phase transferred to the Processing Plate

    b. RNA purification using RNA binding beads

- 50 µL of 100% isopropanol added and shaken for 1 min
- 10 µL of RNA Binding Beads added and shaken for 3 min
- RNA Binding Beads captured magnetically and supernatant discarded
- Washed twice with 150 µL Wash Solution 2 each time
- Beads dried by shaking for 2 min
- RNA eluted in 50 µL of Elution Buffer

2.1.9 Formation of cDNA

From the total RNA isolated, amplified cDNA was made following the Ovation® Pico WTA Systems V2 protocol by NuGEN (http://www.nugen.com/products/microarray-qpcr/ovation-pico-and-picosl-wta-systems-v2).

Procedure overview:

1. First strand cDNA synthesis
    a. 2 µL of First Strand Primer Mix and 5 µL of total RNA were mixed in a 0.2 ml PCR tube
    b. The tube was placed in a thermal cycler running Programme 1 (65 °C, 2 minutes; finally held at 4 °C)
    c. First Strand Mastemix was prepared and 3 µL added to each tube
    d. The tubes were placed in the thermal cycler running Programme 2 (4°C , 2 min; 25°C, 30 min; 42°C , 15 min; 70°C , 15 min; finally held at 4°C)
2. Second strand cDNA synthesis
    a. Second Strand Mastermix was prepared and 10 µL was added to each tube
    b. The tubes were placed in a thermal cycler running Program 3 (4°C, 1 min; 25°C, 10 min; 50°C, 30 min; 80°C, 20 min; finally held at 4°C).
3. Purification of Double-Stranded cDNA
    a. RNAClean® XP beads were re-suspended and brought to room temperature.
    b. 32 µL of beads was added to each tube and mixed. They were then left to incubate at room temperature for 10 minutes. The tubes were placed on the magnet for 5 minutes to completely clear the beads. Only 45 µL of Binding Buffer was removed before the first wash step. The beads were washed while still on the magnet for 30 seconds with 200 µL of freshly prepared 70% ethanol. The wash was repeated 2 more times. Beads were left to dry completely, at least for 15 to 20 minutes.
4. SPIA Amplification
    a. The SPIA Master Mix was prepared by combining SPIA amplification reagents C1 (25 µL/ sample, C2 (50 µL/ sample) and C3 (25 µL/ sample)
    b. 100 µL of the SPIA Master Mix was added to each tube containing the dried beads
    c. The tubes were placed in a thermal cycler running Program 4 (4°C, 1 min; 47°C, 75 min; 95°C, 5 min; finally held at 4ºC)
5. Purification of Amplified SPIA cDNA

2.1.10 Microarray analysis

A microarray is a glass slide with many spots on it where each spot contains thousands of copies of a DNA sequence that uniquely corresponds to a particular gene. The extracted RNA was coloured by different dyes whilst converted to cDNA. This cDNA of the tissue being tested was hybridized onto the slide and each cDNA part of it that represents a gene will bind to its complementary oligonucleotide sequence on the slide. The slide was then excited by laser able to detect the different dyes and the final image was stored for final analysis (http://www.mrc-lmb.cam.ac.uk/genomes/madanm/publications.html).

The isolation of the total RNA, the formation of the cDNA and the analysis of gene expression by microarray was done at the Genomics Centre at King's College London. The candidate was trained in these methods, but only observed work at the Genomics Centre. The microarray was done using the Affymetrix GCS3000 microarray system which uses GeneChip arrays.

The microarray data is MIAME (minimal information about a microarray experiment) compliant and has been deposited in the GEO repository (accession number GSE84954).

The microarray results were analysed using the software Qlucore Omics Explorer version 3.2 and GeneGO Metacore™ (Thomson Reuters).

2.1.11 Relative Quantification Real-Time PCR

Relative quantification real-time PCR was undertaken for the samples of amplified cDNA derived from the tissue samples.

Thirty seven samples of cDNA from 13 participants: 11 liver, 13 muscle and 13 adipose tissue.

The expression of 19 genes was measured in triplicates for each cDNA from each tissue with qRT-PCR:

Table 2-2: The list of genes of the gene expression assays used for the validation of the microarray

Name of Gene
CTGF- Connective tissue growth factor
RGS1- Regulator of G-protein signalling 1
FNDC5- Fibronectin type III domain-containing protein 5
RGS2- Regulator of G-protein signalling
JUN- JUN proto-oncogene
DUSP1- Dual specificity protein phosphatase 1
TGF1- Transforming growth factor 1
JUNB- JUN B proto-oncogene
USF2- Upstream transcription factor 2
ATF3- Activating transcription factor 3
CCL2- C-C Motif Chemokine Ligand 2
EGR1- Early Growth Response 1
PPARA- Peroxisome Proliferator Activated Receptor Alpha
PPARG- Peroxisome Proliferator Activated Receptor Gamma
IL8- Interleukin 8
SOCS3- Suppressor of cytokine signalling 3
IL6- Interleukin 6
LEP- Leptin
GAPDH- Glyceraldehyde 3-P dehydrogenase

For the real time PCR a 384 well plate was used.

Each well had 11 µl of the probe solution (1 µl of gene probe and 10 µl of Taqman® Universal Master Mix II with UNG) and 9 µl of the sample solution (10 ng of cDNA made up to 9 µl of solution by adding RNAse free water).

The plate was sealed and centrifuged at 2000 rpm at 20˚C for 2 minutes and was then placed in the real time machine analyser QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems™). The reactions were carried out using the cycles shown in Table 2-3.

Table 2-3: Real-time PCR cycles

Stage	**Cycles**		**Holding Temp.**	**Holding Time**	**Ramp Rate**
Hold	40		50˚C	2 minutes	Automatic
			95˚C	10 minutes	
PCR Stage			95˚C	15 seconds	
			60˚C	1 minute	

PCR is used to amplify the cDNA. By knowing how much cDNA one has after each cycle of PCR one can calculate how much material there was in the first place, as long as it can be compared to a known standard. The amount after each cycle is measured by using fluorescent coloured dyes that are attached to the PCR products during amplification.

Real time PCR involves 3 steps that are run for 40 cycles and they are:

1: Denaturation- this is done at 95˚C and results in the separation of the 2 DNA strands and therefore ssDNA.

2. Annealing- this is where complementary sequences between the primer and the ssDNA bind- this is done 5˚C below the melting point of the primer.

3. Extension- this done at 70-72 ˚C which is the optimal temperature for the DNA polymerase and the primer extension happens as fast as 100 bases per second.

In relative quantification real-time PCR the expression of the genes of interest are compared between the various samples and the results are expressed as fold change. A 'housekeeping' gene is used as a control for experimental variability- in our experiment this was GAPDH.

Real time PCR analysis curve:

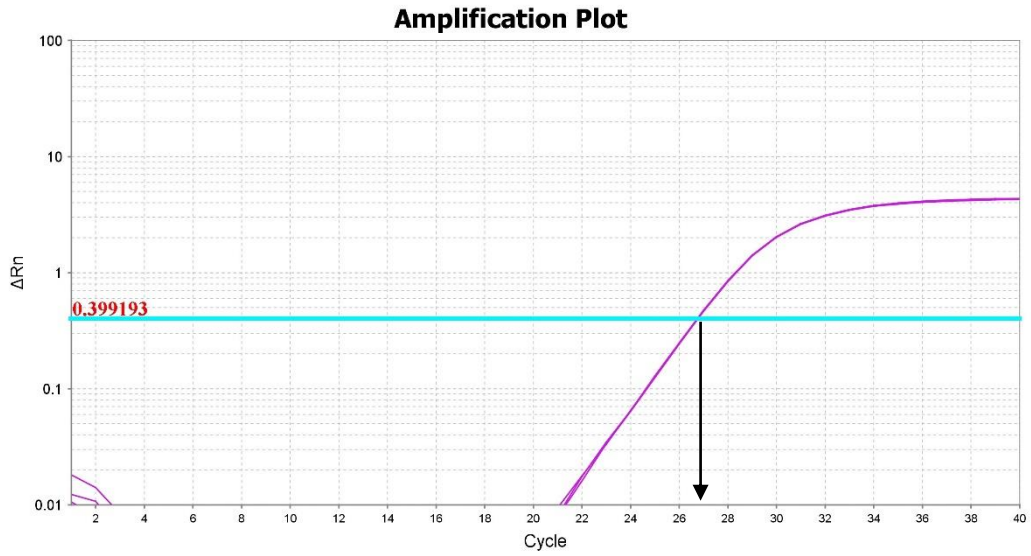

Figure 2.7: Real-time PCR output: calculation of Ct.
Ct threshold cycle, the cyan line is the threshold and the black arrow points to the Ct value.

The real-time PCR output curve (Figure 2.7) has certain important features.

1) The baseline is the signal level, usually during cycles 3-15, where there is little change to the fluorescent signal.
2) The threshold is where the signal is now increased at a statistically significant level above the baseline.
3) The threshold cycle (Ct) is the cycle number during which the reaction signal exceeds the threshold. The higher the template amount the smaller the threshold cycle and vice versa.

In its basic form, in comparative quantification, one takes the Ct values obtained from expression of a gene from the sample of interest and from a control sample- the difference between these 2 values is ΔCt and the fold difference is 2 to the power of ΔCt.

Fold difference = $2^{\Delta Ct.}$

This basic form though does not take into account differences in sample quality, quantity and reaction efficiency. To address these issues the ΔΔCt algorithm is used.

ΔCt sample- ΔCt control sample= ΔΔCt

Where:

ΔCt sample= Ct of the gene of interest in the sample – Ct of the 'housekeeping' gene in the sample

ΔCt control sample= Ct of the gene of interest in the control sample – Ct of the 'housekeeping' gene in the control sample.

For the PCR analysis in this study, Ct of the housekeeping gene was Ct of GAPDH and Ct of the gene of interest in the control sample was the average Ct for that particular gene in the 2 samples from the subjects with Crigler-Najjar type 1 syndrome. The relative quantification real-time PCR was all performed at the Institute of Liver Studies at King's College Hospital by the candidate.

Statistics

Measurements were reported as mean sd scores (standard deviation scores; otherwise known as z scores) and their SD (standard deviation). Comparisons were made between the patients and the healthy controls by performing an independent t-test, or where appropriate, between the measurements of the patients before and after liver transplantation by performing a paired t-test. Linear correlations between variables were tested using the Pearson's correlation coefficient r.

A p value of less than 0.5 was the cut off for statistical significance.

The statistical software used was IBM SPSS Statistics version 22 and for some of the statistical figures GraphPad Prism 7.

For the microarray analysis Qlucore Omics Explorer version 3.2 was used. This software uses p and q values, where "q" is the smallest false discovery rate for which a particular gene would remain on the list of differentially expressed genes. GeneGO Metacore™ software was also used to generate significant networks. These networks have a p value, a z-score and a g-score. The p-value for both software represents the probability of a particular arrangement arising by chance. The z-score ranks the networks according to their saturation in genes coming from the experiment submitted and then the g-score downgrades the z-score depending on if important genes- nodes in the network are not present from the experiment submitted.

All statistical and bioinformatics analysis was done by the candidate.

Chapter 3 RESULTS- "BODY COMPOSITION"

Timeline of Study

The study received R&D approval from King's College Hospital in February 2013 and has been ongoing since. Seventeen children with end stage liver disease undergoing the process of assessment for liver transplantation have been recruited and have had their body composition measured. Two of these children have been removed from the waiting list for a liver transplant whereas 14 of the remaining 15 have now had their liver transplant. Tissue has been collected from thirteen of them and repeat body composition has been performed in nine of them. To date fourteen healthy children have had their body composition measured as controls. Tissue has also been collected from 2 children who were having a liver transplant for Crigler-Najjar type 1 syndrome and this tissue has served as a control for the tissue from the children with cirrhosis and ESCLD.

Recruitment

Seventeen patients with ESCLD being assessed for a liver transplant or already waiting for a liver transplant were recruited and they had their body composition measurements. Ten of these children were female and 7 were male. Age varied form 0.6 years to 17.2 years (mean age 7.4 years and median age 5 years). The diagnoses (Figure 3.1) were biliary atresia (8), Alagille syndrome (2), neonatal sclerosing cholangitis (NSC) (1), a1-antitrypsin deficiency (1), primary sclerosing cholangitis (PSC) (1), cryptogenic cirrhosis (1) and hepatitis C virus (HCV) related cirrhosis (1) as well as two patients who were being assessed for a second transplant (one of these had been originally transplanted for biliary atresia and the other for neonatal haemochromatosis). In terms of ethnic background, 2 patients were Black-African, one was Asian, 1 was mixed race, 1 was white-Greek and the rest were white-British.

Fourteen of the 17 patients who had their body composition measured were transplanted. The age at transplant ranged from 0.65 years to 17.73 years (mean 6.54 years and median 5.3 years). The time that had lapsed from their body composition assessment to the time of their liver transplant ranged from 7 days to almost 2 years (mean 5.3 months and median 6.9 months). As previously mentioned, 2 of the children have been removed from the waiting list.

One of them was a boy with previous biliary atresia, who had already been transplanted, but was being considered for a second liver transplant due to considerable graft dysfunction. The other patient removed was the one with the HCV related cirrhosis. She was treated for the HCV infection and she achieved a sustained viral response after the treatment. As she has been stable from a cirrhosis point of view, she was removed from the liver transplant waiting list. One child from the original 17 is currently still awaiting liver transplant.

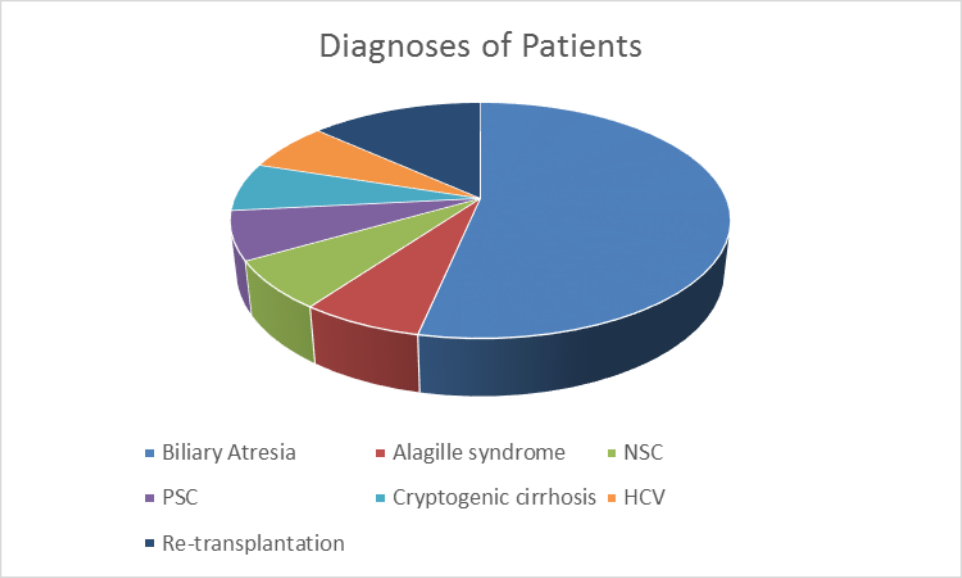

Figure 3.1: Diagnoses of Patients
NSC neonatal sclerosing cholangitis, PSC primary sclerosing cholangitis, HCV hepatitis C virus

Of the 14 patients that have had their liver transplant, 9 have had their second body composition assessment. For these 9 patients the post liver transplant body composition assessment was on average 11.4 months after their liver transplant (range 5.2 to 20 months and median 10.8 months) and on average 15.6 months after their pre liver transplantation body composition assessment (range 10.8 to 21.6 months, median 15.6 months). The child with the HCV related cirrhosis also had a second body composition assessment, 12 months after completion of the HCV treatment and 14 months from her first body composition assessment.

Table 3-1: Blood results of patients at their pre liver transplant body composition assessment

	Mean (SD)	Range	Median
Hb g/L	115.3 (16.7)	81 – 152	116
MCV fL	87.4 (5.8)	75.6 – 95.5	88.7
MCH pg	28.6 (2.6)	23.4 - 33	28.4
MCHC g/L	327.3 (14.6)	306 - 361	327
WCC 10^9/L	6.3 (4.7)	1 – 14.3	4.1
Neutrophils 10^9/L	2.4 (1.6)	0.5 – 6.2	1.8
Lymphocytes 10^9/L	3.3 (3.3)	0.4 – 11.4	1.6
PLTs 10^9/L	106.1 (57.3)	15 - 199	84
Na mmol/L	138.5 (2.6)	133 - 142	139
Urea mmol/L	3.7 (1.7)	1.9 – 8.1	3.2
Creatinine umol/L	25.1 (17.3)	1 - 62	18
Albumin g/L	35 (6.3)	23 - 51	35
Total Protein g/L	64.5 (9.6)	51 - 83	60
INR	1.4 (0.2)	1.03 – 1.85	1.38
Bilirubin umol/L	113.6 (138.2)	16 - 522	57
AST IU/L	154.7 (105.4)	36 – 327	96
ALT IU/L	98.7 (45.7)	33 - 173	100
ALP IU/L	690.6 (488)	229 - 2227	463
GGT IU/L	241.2 (334.9)	15 - 1369	174
TBA mg/dL	117.6 (92.1)	92 - 335	135

SD standard deviation, Hb haemoglobin, MCV mean corpuscular volume, MCH mean corpuscular haemoglobin, MCHC mean corpuscular haemoglobin concentration, WCC white cell count, PLTs platelets, Na sodium, INR international normalised ratio, AST aspartate aminotransferase, ALT alanine transaminase, ALP alkaline phosphatase, GGT gamma-glutamyl transpeptidase, TBA total bile acids.

Out of the 17 patients, 9 were anaemic with a haemoglobin below the lowest normal range for their age, and two of the patients had a low MCV and MCH. Nine patients had a higher than normal MCV (3 of which with a higher MCH as well), but only 2 of these also had a low haemoglobin. Nine patients had a lower than normal white cell count and all patients had a below normal platelet count. In terms of liver synthetic function, 7 of the patients had below

normal albumin levels with 1 patient having higher than the normal range levels (the one with Alagille syndrome). INR was prolonged in 14 patients. All the patients were cholestatic. All of the patients had splenomegaly with evidence of hypersplenism. Three patients had experienced a variceal GI bleed prior to being transplanted. Two of the patients were diagnosed with hepatopulmonary syndrome. One of them requiring oxygen in the final stages prior to her liver transplant (this was the patient with the NSC). In addition, one of the patients being assessed for her second liver transplant had adrenal insufficiency.

All children received dietetic input as part of their ongoing management. The child with the NSC was prescribed oral high calorie supplements, but with poor compliance and the family refused nasogastric feeding. One of the children with Alagille syndrome was on overnight feeds via gastrostomy and one of the young girls with biliary atresia was supplementing her oral intake with nasogastric feeds.

As mentioned earlier 14 healthy controls (8F: 6M) also had body composition measurements. Their ages varied from 8 months to 18 years old (mean 7.35 years, median 5.5 years). These children had no medical issues.

Results by body composition method

The following 2 tables summarise the body composition methods performed on the patients. The results are in the first instance reported as per method.

Table 3-2: Body composition methods for the patients under 4 years of age

Body composition methods for the patients under 4 years of age
1. Basic anthropometry; weight, height and BMI
2. MUAC and Skinfold thicknesses :triceps, biceps and subscapular
3. Deuterium dilution studies
4. Bromide dilution studies
5. Bioelectrical impedance
6. Indirect calorimetry

BMI body mass index, MUAC mid upper arm circumference

Table 3-3: Body composition methods for the patients over 4 years of age

Body composition methods for the patients over 4 years of age
1. Basic anthropometry; weight, height and BMI
2. MUAC and Skinfold thicknesses :triceps, biceps and subscapular
3. Deuterium dilution studies
4. Bromide dilution studies
5. Whole body DXA
6. Air displacement plethysmography (BOD POD)
7. 3D photonic scanning
8. Bioelectrical impedance
9. Indirect calorimetry

BMI body mass index, MUAC mid upper arm circumference, DXA dual energy X-ray absorptiometry

Table 3-4: Body composition methods for the healthy controls under 4 years of age

Body composition methods for the healthy controls under 4 years of age
1. Basic anthropometry; weight, height and BMI
2. MUAC and Skinfold thicknesses :triceps, biceps and subscapular
3. Deuterium dilution studies
4. Bioelectrical impedance
5. Indirect calorimetry

BMI body mass index, MUAC mid upper arm circumference

Table 3-5: Body composition methods for the healthy controls over 4 years of age

Body composition methods for the healthy controls over 4 years of age
1. Basic anthropometry; weight, height and BMI
2. MUAC and Skinfold thicknesses :triceps, biceps and subscapular
3. Deuterium dilution studies
4. Air displacement plethysmography (BOD POD)
5. Bioelectrical impedance
6. Indirect calorimetry

BMI body mass index, MUAC mid upper arm circumference

The following 2 tables show the number of patients and healthy controls that were recruited and the elements of the body composition they participated in.

Table 3-6: Number of patients participating in body composition studies

Patients			
	11 were > 4 years old	**6 were < 4 years old**	**Total**
Basic anthropometry	11	6	17
MUAC/skinfolds	10	5	15
Deuterium	11	6	17
Bromide	8	4	12
DXA	11	N/A	11
BOD POD	10	N/A	10
3D photonic scanning	4	N/A	4
BIA	11	6	17
Indirect calorimetry	11	6	17

BIA bioelectrical impedance, BOD POD air displacement plethysmography, DXA dual energy X-ray absorptiometry, MUAC mid upper arm circumference

Table 3-7: Number of healthy controls participating in body composition studies

Healthy Controls			
	10 were > 4 years old	**4 were < 4 years old**	**Total**
Basic anthropometry	10	4	14
MUAC/skinfolds	10	4	14
Deuterium	10	4	14
Bromide	N/A	N/A	N/A
DXA	N/A	N/A	N/A
BOD POD	10	N/A	10
3D photonic scanning	N/A	N/A	N/A
BIA	10	4	14
Indirect calorimetry	10	3	13

BIA bioelectrical impedance, BOD POD air displacement plethysmography, DXA dual energy X-ray absorptiometry, MUAC mid upper arm circumference

The healthy controls had only one body composition assessment. The patients however were also assessed at least 6 months after their liver transplant. Out of the 17 patients, 14 were transplanted, 2 were taken off the transplant list and one is still awaiting transplant. Of the 14 transplanted ones, 2 were lost to follow up as they lived abroad. 9 of the 12 remaining patients had a post transplantation body composition assessment at the time of this thesis. Out of the 2 who were taken off the waiting list, one had a second assessment after completing treatment for HCV infection and the other did not have a full assessment, but had a whole body DXA scan.

Table 3-8: Number of patients participating in body composition studies after transplant or treatment

Patients after transplant or after treatment			
	10 were > 4 years old	**4 were < 4 years old**	**Total**
Basic anthropometry	5	5	10
MUAC/skinfolds	5	3	8
Deuterium	5	5	10
Bromide	5	3	8
DXA	6	N/A	6
BOD POD	5	N/A	5
3D photonic scanning	4	N/A	4
BIA	5	5	10
Indirect calorimetry	5	5	10

BIA bioelectrical impedance, BOD POD air displacement plethysmography, DXA dual energy X-ray absorptiometry, MUAC mid upper arm circumference

3.1.1 Anthropometry

The weight, height and BMI measurements were converted to sd scores using the UK WHO data in ImsGrowth program© (Cole, 1990). The database is a combination of WHO and UK 1990 to age 4 years and UK 1990 from 4 years to 20 years of age (full term children) (Freeman et al., 1995, de Onis et al., 2006). We have weight, height and BMI sd.scores on 17 patients (7M:10F) and 14 healthy controls (6M:8F). Six of the patients and 4 of the healthy controls were under the age of 4 years.

Looking at the table below we see that when the patient and the control group are compared as a whole, the patients have significantly lower sd scores for weight and height, but when the comparison takes sex into account these differences diminish.

Table 3-9: Mean weight, height and BMI sd scores for patients and controls

	Number	**Age (years)**	**Mean Weight sds (SD)***	**Mean Height sds (SD)****	**Mean BMI sds (SD)**
Patients	17	7.39 (6.0)	-0.88 (0.85)	-1.15 (1.54)	-0.21 (0.9)
Controls	14	7.35 (5.4)	0.23 (1.31)	0.18 (1.29)	0.14 (1.12)

*the difference of mean weight s.ds between the 2 groups reached statistical significance at $p < 0.01$
**the difference of mean height s.ds between the 2 groups reached statistical significance at $p < 0.05$
BMI body mass index, sds standard deviation score, SD standard deviation

Table 3-10: Mean weight, height and BMI sd scores for patients and controls according to sex

Patients	**Mean Weight sds (SD)**	**Mean Height sds (SD)**	**Mean BMI sds (SD)**
Male	-0.88 (1.05)	-1.42 (1.22)	0.79 (0.83)
Female	-0.87 (0.74)	-0.96 (1.77)	-0.42 (0.93)
Controls			
Male	-0.53 (1.27)	-0.39 (0.98)	-0.5 (1.15)
Female	0.81 (1.08)	0.61 (1.38)	0.62 (0.89)

BMI body mass index, sds standard deviation score, SD standard deviation

The difference of the mean weight, height and BMI sd score between patients and controls for male and female participants tested separately with a 2-tailed independent t-test did not achieve statistical significance in any group.

Table 3-11: Mean weight, height and BMI sd scores for patients and controls divided by age

Patients	**Mean Weight sds (SD)**	**Mean Height sds (SD)**	**Mean BMI sds (SD)**
Under 4 years old	-1.12 (0.55)*	-1.45 (0.83)**	-0.36 (0.81)
Over 4 years old	-0.74 (0.97)	-0.99 (1.84)	-0.13 (0.97)
Controls			
Under 4 years old	0.895 (1.42)*	1.13 (1.4)**	0.4 (1.24)
Over 4 years old	-0.03 (1.24)	-0.2 (1.08)	0.04 (1.12)

*The difference of the mean weight sd score between patients and controls under the age of 4 years was statistically significant $p < 0.05$
** The difference of the mean height sd.score between patients and controls under the age of 4 years was statistically significant $p < 0.01$.
The other comparisons did not reach statistical significance.
BMI body mass index, sds standard deviation score, SD standard deviation

When comparing the mean sd scores of our patients to an sd score of 0, the difference reaches statistical significance for weight at $p < 0.001$ and for height at $p < 0.01$, but not for BMI. When the same is done for our healthy controls none of the differences are statistically significant.

In summary, overall the patients are significantly underweight and short, with this being more significant in the under 4 years of age children.

The average of 3 measurements of MUAC and skinfold thickness measurements recorded for each patient and each healthy control was converted to the corresponding sd score. For the under 4 year olds this was done using the WHO database and for the over 4 year olds this was done using the study population previously measured by Wells et al. The WHO database does not include reference values for biceps skinfold thickness for the young children.

Table 3-12: Mean sd score for MUAC, triceps and subscapular skinfold for the children under 4 years of age

	Patients (5)	**Controls (4)**	**P value**
MUAC	-1.1340 (0.49)	1.2 (0.51)	0.0002
Triceps	-1.34 (1.12)	0.11 (0.93)	NS
Subscapular	-1.68 (1.73)	1.49 (2.29)	NS (0.06)

MUAC mid upper arm circumference, in brackets the standard deviation

Table 3-13: Mean sd scores for MUAC, triceps, biceps and subscapular skinfolds for children over 4 years of age

	Patients (10)	**Controls (10)**	**P value**
MUAC	-1.13 (0.69)	0.02 (0.86)	0.0048
Triceps	-0.32 (0.89)	-0.14 (1.19)	NS
Biceps	-0.45 (0.54)	0.47 (0.81)	0.0085
Subscapular	0.07 (0.79)	0.21 (1.1)	NS

MUAC mid upper arm circumference, in brackets the standard deviation

For nine of the patients that have had their liver transplant we have anthropometry measurements post liver transplant.

Table 3-14: Mean weight, height and BMI sd scores before and after liver transplantation.

	Mean Weight sds (SD)	Mean Height sds (SD)	Mean BMI sds (SD)
Pre-transplant	-0.84 (0.88)	-1.26 (0.97)	-0.14 (0.82)
Post-transplant	-0.25 (1.29)	-0.9 (1.13)	0.46 (0.93)

BMI body mass index, sds standard deviation score, SD standard deviation

There was an increase in weight, height and BMI sd scores for these 9 patients, but this difference in sd scores before and after transplant did not reach statistical significance when applying a paired t-test.

Height after transplant correlated strongly with height before transplant ($r=0.84$, $p <0.05$), this was not the case for weight and BMI.

Table 3-15: Mean MUAC, triceps, biceps and subscapular skinfolds before and after liver transplantation for children over 4 years of age

	Pre-transplant	Post-transplant	P value
MUAC	-1.07 (0.75)	-0.12 (0.66)	0.000
Triceps	0.32 (0.56)	1.0 (0.49)	0.099
Biceps	-0.45 (0.82)	0.9 (0.75)	0.057
Subscapular	-0.11 (0.53)	0.68 (0.55)	0.014

MUAC mid upper arm circumference

We have anthropometry data for 4 children who were older than 4 years of age before and after liver transplantation. MUAC, biceps, triceps and subscapular skinfolds all increased after the transplant. This increase reached statistical significance for MUAC and subscapular skinfold. The younger children did not cooperate consistently with the skinfold measurements post liver transplant, so we can only compare MUAC before and after transplant in this age group. Even though there was an increase in mean MUAC sd score from -1.7 to -0.9, this did not reach statistical significance.

In summary, the patients under 4 years of age had an MUAC sd score significantly less than the controls, whereas for the older children it was MUAC and biceps skinfold that was less. After

transplant, all indices increased for the older children, but only the MUAC and subscapular skinfolds sd scores increased significantly. Height after transplantation correlated with height before transplantation.

3.1.2 Deuterium Dilution Studies

All participants had deuterium dilution studies as part of their body composition assessment in order to assess TBW. The patients had TBW measured by the equilibrium or plateau method and the healthy controls by the back extrapolation method. The samples as previously mentioned were analysed in batches.

TBW is mostly found in fat free mass. By using the Lohman hydration factor, TBW can be converted to FFM, where TBW (kg) = hydration factor X FFM (kg) (Hewitt et al., 1993). The results were assigned the appropriate sd score using data from a reference population of healthy children (Wells et al., 2012, Wells et al., 2010b)

All 17 patients have had their deuterium analysed for the before liver transplant body composition and subsequently their TBW calculated. Looking at the first 9 patients, we can see that for 3 of the younger ones, the results generated are not plausible (numbers in italics). FM takes a negative value and therefore the results are invalid. This was thought to be due to the infants' reluctance to drink the solution, resulting in a degree of drooling making it difficult to assess the exact amount of dose taken by the children. Further to this, 2 of the older children, (results underlined) also most likely have inaccurate results, this was thought to be due to suboptimal mixing of the solution. At the time the deuterium and bromide solutions were given together as one to the children. Because of these issues, for all subsequent measurements, the deuterium was given separately to the bromide solution and extra care was taken to mix the solutions well, particularly prior to taking out the sample of the solution. After these measures, the results were satisfactory for subsequent participants.

Table 3-16: TBW results for the patients prior to liver transplantation

ID	Age	Sex	Wt	TBW	sds	HF %	LM	LM %	FM	FM %
1	118	F	31.8	20.9	0.61	77	27.14	85.35	4.7	14.65
2	20	M	11.04	5.79	-2.88	78.6	7.37	66.72	3.7	33.28
3	62	M	21.7	8.33	-3.6	77	10.82	49.85	10.9	50.15
4	7	F	7.14	*7.04*	*4.79*	*78.8*	*8.93*	*125.13*	*-1.8*	*-25.13*
6	58	M	15.2	8.2	-3.42	77.8	10.54	69.34	4.7	30.66
7	57	F	15.45	9.22	-1.14	78.3	11.78	76.22	3.7	23.78
8	10	M	8.08	*6.55*	*1.2*	*79*	*8.29*	*102.61*	*-0.2*	*-2.61*
9	36	F	11.4	2.22	-8.67	78.3	2.84	24.87	8.6	75.13
10	8	F	6.98	*0*	*n/a*	*78.8*				
11	126	F	33.3	20.11	-0.17	77	26.12	78.43	7.2	21.57
12	12	F	7.06	4.9	0.92	78.5	6.24	88.41	0.8	11.59
13	181	F	42.4	21.34	-2.52	77	27.71	65.36	14.7	34.64
14	112	M	21.8	12.45	-3.2	76.2	16.34	74.95	5.5	25.05
15	206	M	54.26	32.04	-1.53	74.2	43.18	79.58	11.1	20.42
16	200	F	59.01	30.76	-0.16	75	41.01	69.50	18.0	30.50
17	104	F	24.53	14.48	-0.83	77.6	18.66	76.07	5.9	23.93
18	188	M	53.62	35.23	-0.47	74.2	47.48	88.55	6.1	11.45

Age in months, Wt weight in kilograms, TBW total body water in kilograms, sds standard deviation score, HF hydration factor, LM lean mass in kilograms, FM fat mass in kilograms

Fourteen of the healthy children that had their body composition also had deuterium studies. For 2 of these their results have not been analysed yet. The results of the remaining 12 can be seen in the following table. The second child had invalid results due to the solution not being mixed well enough (results in italics), but subsequent results were satisfactory.

Table 3-17: TBW results for the healthy controls

ID	Age	Sex	Wt	TBW	SDS	HF %	LM	LM %	FM	FM %
C1	39	M	13.2	7.99	-1.52	77.8	10.27	77.80	2.93	22.20
C2	55	M	19.25	*0.04*	*n/a*	*77.8*				
C6	9.5	F	9.2	5.06	1.14	78.8	6.42	69.80	2.78	30.20
C5	54	M	17.68	11.7	0.5	78.3	14.94	84.52	2.74	15.48
C7	120	M	29	16.77	-1.25	76.2	22.01	75.89	6.99	24.11
C8	162	F	38.85	22.02	-1.03	75.5	29.17	75.07	9.68	24.93
C9	116	M	32.33	14.09	-2.36	76.2	18.49	57.19	13.84	42.81
C10	216	F	69.14	32.42	0.15	75	43.23	62.52	25.91	37.48
C11	190	M	38.76	27.01	-2.06	74.2	36.40	93.92	2.36	6.08
C12	52	F	19.78	12.63	1.72	78.3	16.13	81.55	3.65	18.45
C13	78	F	25.15	13.98	0.55	78	17.92	71.26	7.23	28.74
C14	115	F	26.36	13.1	-2.3	77	17.01	64.54	9.35	35.46

Age in months, Wt weight in kilograms, TBW total body water in kilograms, Sds standard deviation score, HF% hydration factor %, LM lean mass in kilograms, FM fat mass in kilograms

Nine children had deuterium dilution studies post liver transplantation, as did the child who was taken off the transplantation list and was treated for HCV infection. For the child with the HCV infection and for 1 of the post liver transplant children, their deuterium samples have not been analysed yet. The results of the remaining 8 children can be seen below.

Table 3-18: Results of TBW post liver transplant

ID	Age	Sex	Wt	TBW	sds	HF %	LM	LM %	FM	FM %
3	84	M	26.13	14.81	0.1	76.8	19.28	73.80	6.85	26.20
4	19	F	13.56	0	n/a					
8	21	M	12.12	8.68	1.06	78.6	11.04	91.12	1.08	8.88
9	52	F	17.06	8.78	-1.15	78.3	11.21	65.73	5.85	34.27
10	24	F	10.7	7.62	0.34	78.5	9.71	90.72	0.99	9.28
12	28	F	9.68	6.17	-1.89	78.5	7.86	81.20	1.82	18.80
15	219	M	53.8	29.26	-2.22	74.2	39.43	73.30	14.37	26.70
17	115	F	25.29	13.4	-2.15	77	17.40	68.81	7.89	31.19

Age in months, Wt weight in kilograms, TBW total body water in kilograms, Sds standard deviation score, HF% hydration factor %, LM lean mass in kilograms, FM fat mass in kilograms

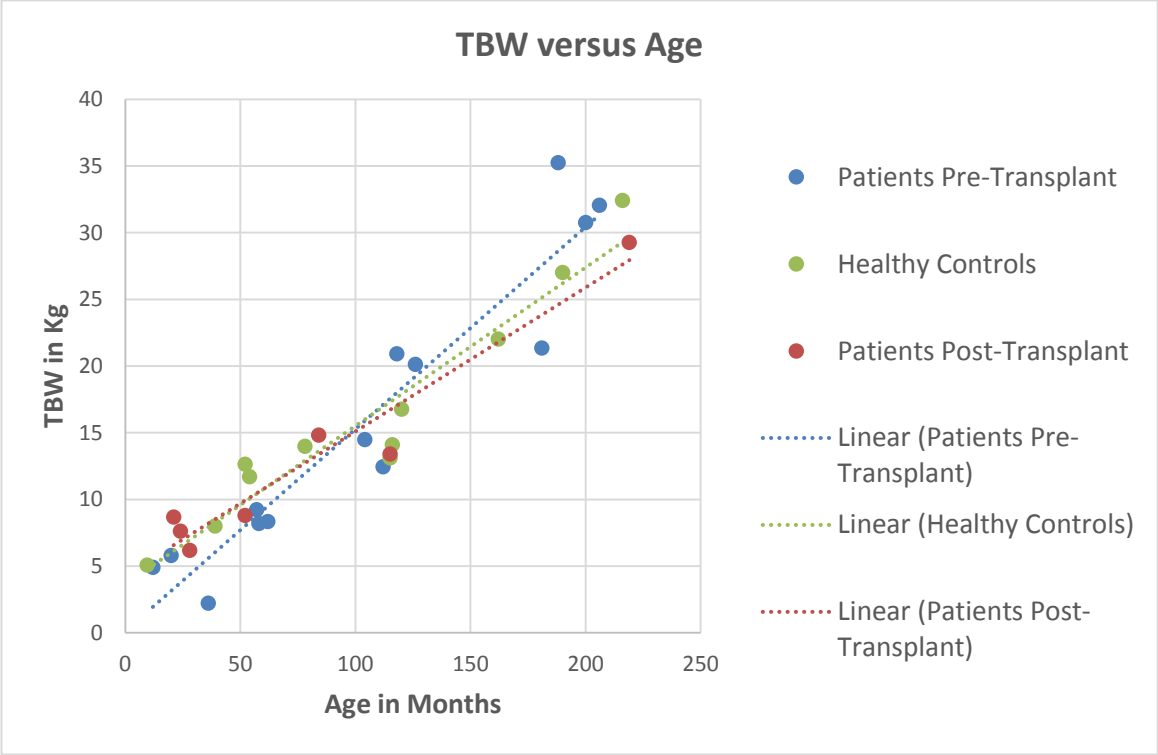

Figure 3.2: TBW (in kg) versus Age in Months
TBW total body water

TBW increased with age, as can be seen in Figure 3.2 for all groups.

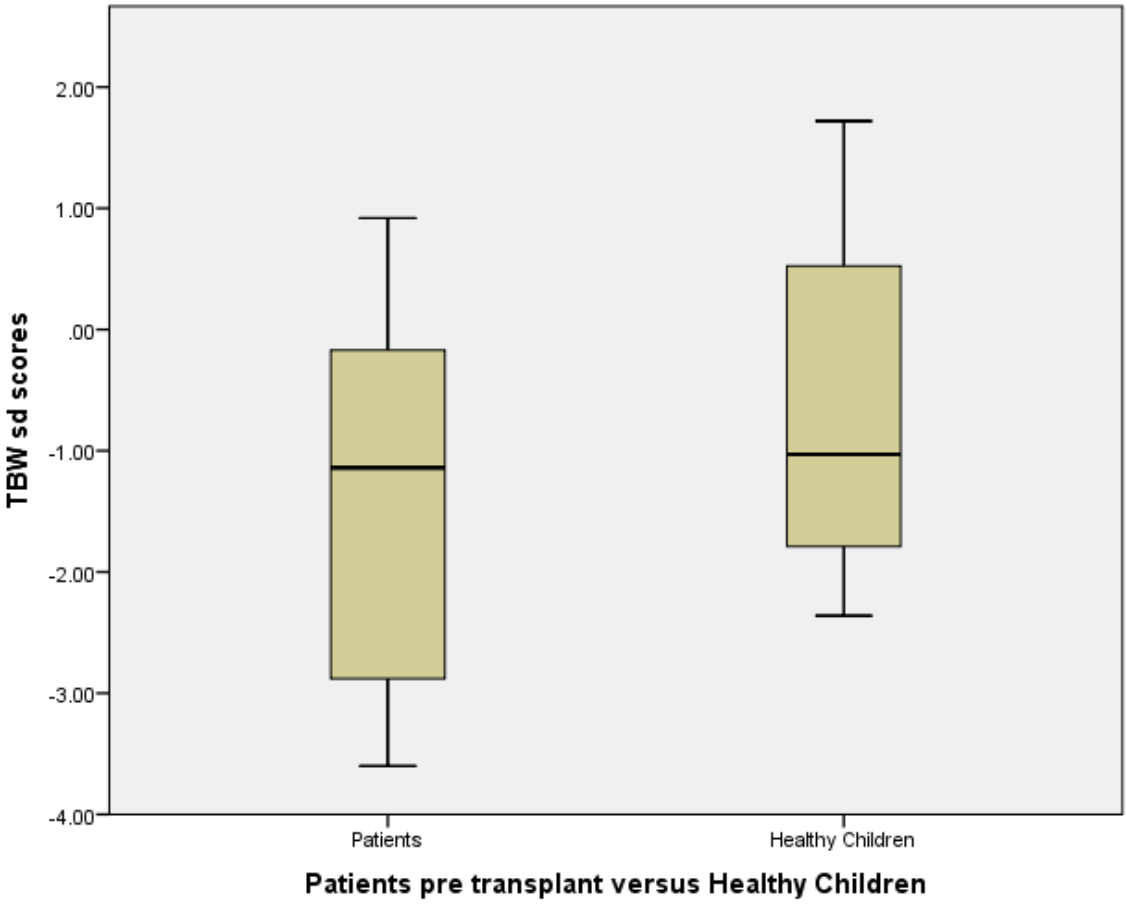

Figure 3.3: Box plots of sd scores for measured TBW of patients before liver transplant and of healthy controls
TBW total body water, sd standard deviation

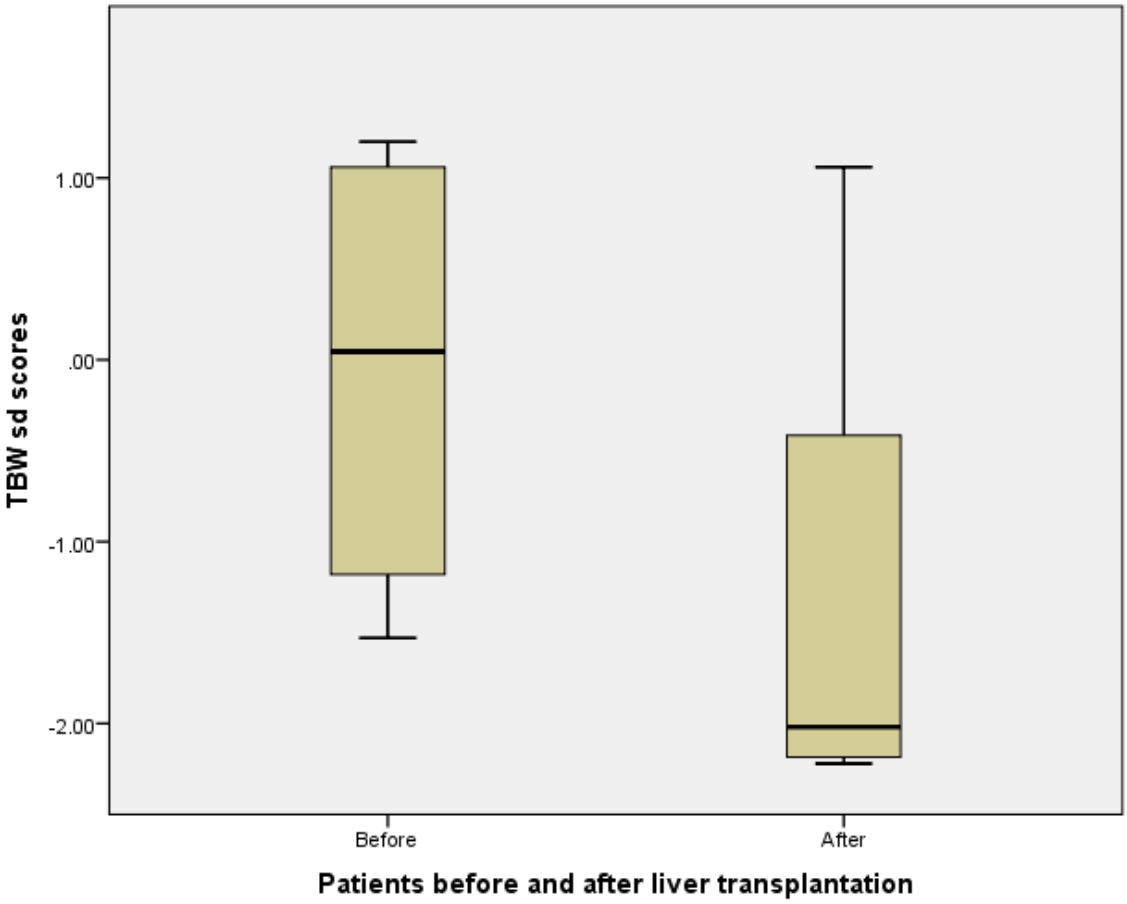

Figure 3.4: Box plots of sd scores for TBW of patients before and after liver transplantation.
TBW total body water, sd standard deviation

The difference in mean sd score for TBW between the patients and the healthy controls (Figure 3.3) did not achieve statistical significance. When the mean sd scores for TBW of these 2 populations were tested individually against an sd score of zero with a paired t-test, the patients did have a significant difference (-1.41 ± 1.56, $p < 0.05$) whereas the healthy children did not (-0.59 ± 1.45, $p = 0.2$).

After liver transplantation there was a reduction in the mean sd score for TBW, but the difference in mean sd score between the patients before liver transplant and after liver transplant did not achieve statistical significance (1.24 ± 1.15, $p=0.12$).

Various prediction equations exist to predict TBW for healthy children. Wells et al (Wells et al., 2005) have published a set of these equations for boys and girls, taking into account age, weight and height and based on a UK population. These equations are:

For the boys:

Ln(TBW)= -2.952 + 0.551*Ln(Wt) +0.796*Ln(Ht) +0.008(Age),

Where Ln(TBW) in the natural logarithm of the total body water in litres, Wt is weight in kilograms, Ht is height in centimetres and age is in years.

For the girls, in a similar way:

Ln(TBW)= -2.952 + 0.551*Ln(Wt) +0.796*Ln(Ht) -0.047 +0.008(Age).

Using the above equations, the predicted TBW for each participant was calculated as was the average TBW (the average of the measured and the predicted TBW) and the values were plotted against the percent difference of the measured TBW minus the predicted TBW. One can see that there is agreement between measured and predicted TBW for the healthy children, but not for the patients either before or after liver transplantation.

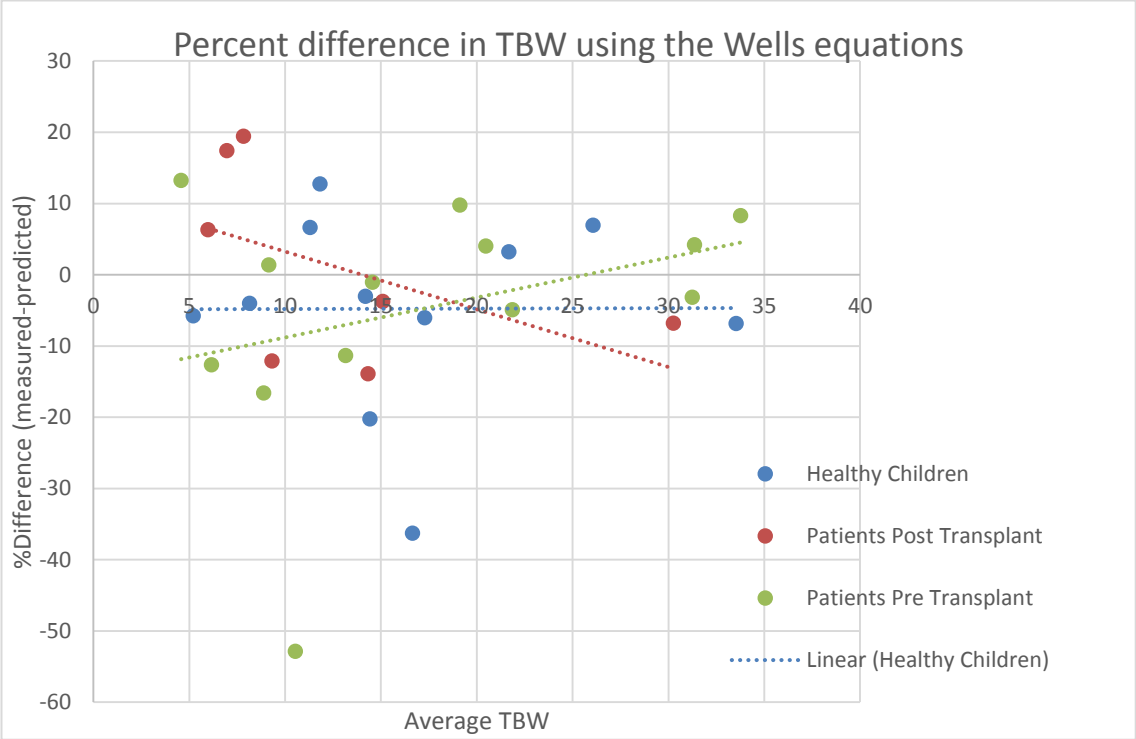

Figure 3.5: Percent difference in TBW using the Wells equations for patients before and after liver transplant and healthy children
TBW total body water

In summary, from the 12 patients with valid deuterium results, 4 of them had an sd score < 1.96 (<5th centile) for TBW. None of the children had a FM <10%. From the 11 healthy children, 3 had an sd score < 1.96 and one of them also had a FM <10%. After the transplant, for 7 of the patients TBW sds was < 1.96 for 2 of them and FM < 10% for another 2.

TBW sds scores were not different between patients and healthy controls. TBW may be an indirect measure of lean mass, but this would include organomegaly in the patients and of course the TBW would also include water retention or oedema the patients may have. TBW sds scores reduced post liver transplant, but this reduction did not achieve statistical significance. The prediction equation for TBW by Wells et al (Wells et al., 2005) worked well for the healthy children, but not for the patients.

3.1.3 Bromide Dilution Studies

According to the protocol, only the patients and not the healthy children had bromide dilution studies. The bromide solution was of a larger volume to the deuterium and therefore more children refused to drink it. It also has a salty taste, which some of the children disliked.

Of the 17 patients, 5 did not have bromide dilution studies and of the 12 remaining, 7 had valid results. The children with the invalid results (results in italics) were the same as the ones with the problematic results from the deuterium studies.

Post liver transplant, of the 9 patients eligible to have the test, 2 refused and therefore 7 of them completed the test. In addition the child treated with HCV infection also had the bromide dilution study. Her samples, as well as for 2 of the patients are still to be analysed. We therefore have results from 5 patients that were post their liver transplant, all valid.

Unfortunately the 7 patients with valid results pre liver transplant and the 5 with results so far post liver transplant could not be paired, so were compared as independent groups.

Table 3-19: ECW estimated by bromide dilution before and after liver transplantation.

ID	PreTx-Age	Wt (kg)	ECW (kg)	ECW%/Wt
1	118	31.8	9.23	29.04
2	20	11.04	3.07	27.77
3	62	*21.7*	*11.64*	*53.62*
4	7	*7.14*	*0.50*	*6.96*
6	58	*15.2*	*8.75*	*57.57*
7	57	15.45	3.71	24.02
8	10	*8.08*	*7.57*	*93.69*
10	8	*6.98*	*0*	*0*
11	126	33.3	9.58	28.77
14	112	21.8	5.47	25.08
15	206	54.26	6.18	25.18
18	188	53.62	16.31	30.42
ID	**PostTx-Age**	**Wt (kg)**	**ECW (kg)**	**ECW%/Wt**
3	84	26.13	6.18	23.65
9	52	17.06	3.95	23.13
10	24	10.7	2.57	24.06
12	28	9.68	2.64	27.27
15	219	53.8	12.28	22.82

PreTx before transplant, PostTx after transplant, Wt weight, kg kilograms, ECW extra cellular water

The difference between the mean ECW% per kg of BW before liver transplant (27.18% +/- 2.42) was significantly different to the mean post liver transplant (24.19% +/- 1.79 kg) when compared with an independent t-test ($p<0.05$), as shown in Figure 3.6.

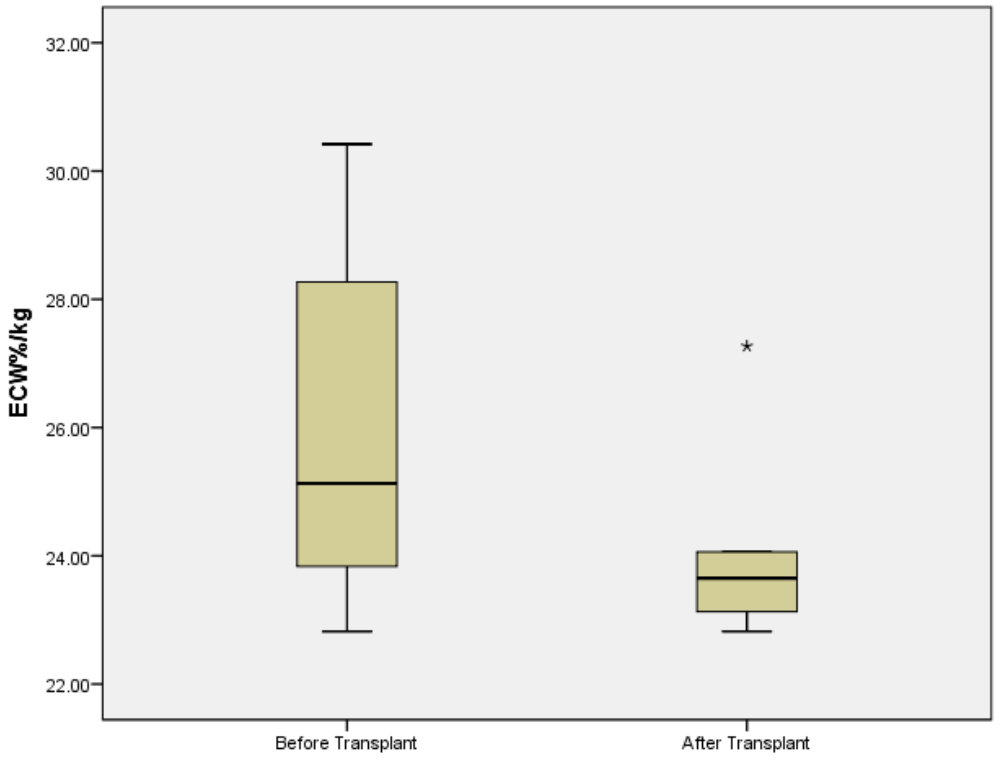

Figure 3.6: Percentage of ECW per kilogram of body weight before and after liver transplant
ECW extra cellular water, kg kilograms

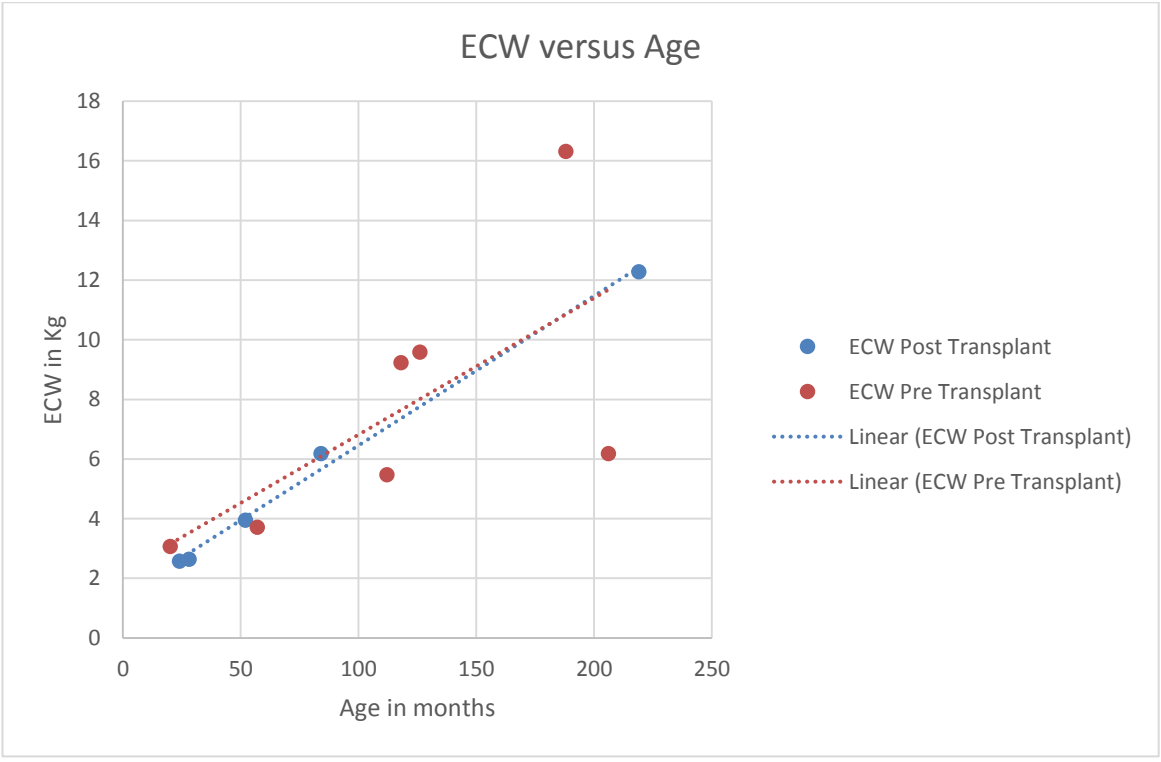

Figure 3.7: ECW versus age before and after liver transplantation
ECW extra cellular water, kg kilograms

The measurement of TBW and ECW allows as to calculate intracellular water (ICW), which is TBW minus ECW.

Table 3-20: ICW and ECW:ICW before and after liver transplantation

Pre Tx- ID	**TBW (lit)**	**ECW (lit)**	**ICW (lit)**	**ECW:ICW**
1	21.03	9.29	11.74	0.79
2	5.83	3.08	2.74	1.13
6	8.25	3.73	5.54	0.67
11	20.11	9.64	10.6	0.91
14	12.45	5.5	7.03	0.78
17	14.48	6.2	8.36	0.74
18	35.23	16.42	19.04	0.86
Post Tx- ID	**TBW (lit)**	**ECW (lit)**	**ICW (lit)**	**ECW:ICW**
3	14.9	6.22	8.68	0.72
9	8.84	3.97	4.86	0.82
10	7.67	2.59	5.08	0.51
12	6.21	2.66	3.55	0.75
15	29.45	12.28	17.09	0.72

PreTx before transplant, PostTx after transplant, TBW total body water, ECW extracellular water, ICW intracellular water, lit litres

The difference of the mean ECW:ICW before liver transplant (0.79 +/- 0.085) and the mean ECW:ICW after liver transplant (0.70 +/- 0.116) did not reach statistical significance.

In summary, the bromide dilution studies were the most challenging. From the results we have, ECW expressed as % per BW significantly reduced post liver transplant, but this was not reflected in a reduction of the ratio ECW: ICW.

3.1.4 Air displacement plethysmography (BOD POD)

According to the protocol, all children healthy and patients, were to have the BOD POD if they were over 4 years of age. All children over 4 years of age attempted the BOD POD. Eleven of the patients were over 4 years of age, but one of them refused to enter the BOD POD. Of the remaining 10 patients, two of the younger ones were not able to yield consistent results. We

therefore have valid measurements from 8 of the older patients. Of the 14 healthy controls, 10 were over 4 years of age and they all attempted the BOD POD with success.

BOD POD gives us raw body volume measurements. Raw body volume was adjusted using age and sex appropriate predicted residual lung volume (Rosenthal et al., 1993, Zapletal et al., 1976) and surface area artefact (Haycock et al., 1978) to calculate the actual body volume (ABV). Body weight (BW) divided by ABV gives total body density (TBD), which is then converted to fat mass percentage by using constants C1 and C2 from Wells et al (Wells et al., 2010b).

TBD = BW/ARV

FM% = [(C1/BD)-C2]*100

FM = BW * (FM%/100)

FFM = BW – FM

FM and FFM indices were converted to sd scores (z scores) using the previously mentioned reference population.

By looking at the patients, we see 5 out of eight have a fat mass percentage of over 20% and 3 of them over 30%. In spite of this their fat mass sd score for their age and sex is within normal limits. This could reflect a reduction in lean mass, which is really why proportionally the fat mass seems more. In fact 3 children have significantly low fat free mass sd scores.

In healthy individuals, BMI tends to reflect fat mass and frequently fat mass correlates with fat free mass. In the patients, there was no significant correlation between BMI sd score and FM sd score, FM sd score and FFM sd score, FM percentage and BMI and FM percentage and BMI sd score for the patients.

By looking at the healthy children, we see 7 out of ten have a fat mass percentage of over 20%, and 1 of them over 30%. Again FM sd scores are within normal limits, apart from one child with a very low FM sd score and also a very low BMI sd score. There is though now an important distinction. In the healthy children BMI sd score and FM sd score correlate strongly and have a Pearson's correlation factor of 0.886 (p= 0.001). FM sd score and FFM sd score also correlate significantly (r= 0.678, p = 0.031). The correlation between FM percentage and BMI doesn't quite reach significance (r=0.63, p=0.051), but between FM percentage and BMI sd score it does (r=0.765 and p=0.01).

There was no significant difference between any of the indices when compared between the 2 groups.

Table 3-21: Indices from the BOD POD for the patients

ID	Age	Sex	BMI sds	FM	FM%	FFM	TBD	FM sds	FFM sds	TBD sds
1	9.89	F	**-2.21**	5.68	17.87	26.12	1.051	-0.87	0.14	0.95
3	5.16	M	1.18	5.38	24.77	16.32	1.031	1.1	-0.15	-1.08
11	10.54	F	0.86	8.09	24.27	25.23	1.038	-0.3	-0.58	0.11
14	9.31	M	-0.58	3.18	14.6	18.62	1.056	-1.14	**-1.96**	0.63
15	17.17	M	0.27	18.63	34.34	35.63	1.022	0.91	**-3.22**	-1.63
16	16.69	F	0.12	20.56	34.85	38.45	1.023	0.66	-0.88	-0.73
17	8.7	F	-0.44	8.03	32.72	16.5	1.02	0.12	**-2.32**	-0.89
18	15.7	M	-1.04	8.25	15.38	45.37	1.06	-0.2	-0.95	-0.08

BOD POD air displacement plethysmography, Age in years, M male, F female, BMI body mass index, sds standard deviation score, FM fat mass, FFM fat free mass, TBD total body density

Table 3-22: Indices from the BOD POD for the healthy controls

ID	Age	Sex	BMI sds	FM	FM%	FFM	TBD	FM sds	FFM sds	TBD sds
C2	4.56	M	0.22	2.47	12.85	16.78	1.055	-0.33	0.68	**-2.47**
C5	4.51	M	-0.37	3.67	20.75	14.01	1.039	0.57	-0.72	**-2.67**
C7	10.06	M	-0.09	4.7	16.21	24.3	1.054	-0.48	-0.53	0.66
C8	13.48	F	0.55	10.21	26.28	28.64	1.036	-0.21	-1.26	0.01
C9	9.69	M	0.54	9.26	28.64	23.07	1.028	0.86	-0.66	-1.1
C10	17.94	F	1.26	23.46	33.93	45.68	1.025	0.81	0.54	-0.62
C11	15.84	M	**-2.69**	2.32	5.98	36.44	1.081	**-2.53**	**-2.45**	1.43
C12	4.32	F	1.09	4.96	25.06	14.82	1.031	0.65	0.73	-0.31
C13	6.53	F	0.46	6.39	25.40	18.76	1.035	0.3	0.32	-0.07
C14	9.6	F	-0.61	7.19	27.26	19.17	1.032	-0.32	-1.86	-0.27

BOD POD air displacement plethysmography, Age in years, M male, F female, BMI body mass index, sds standard deviation score, FM fat mass, FFM fat free mass, TBD total body density

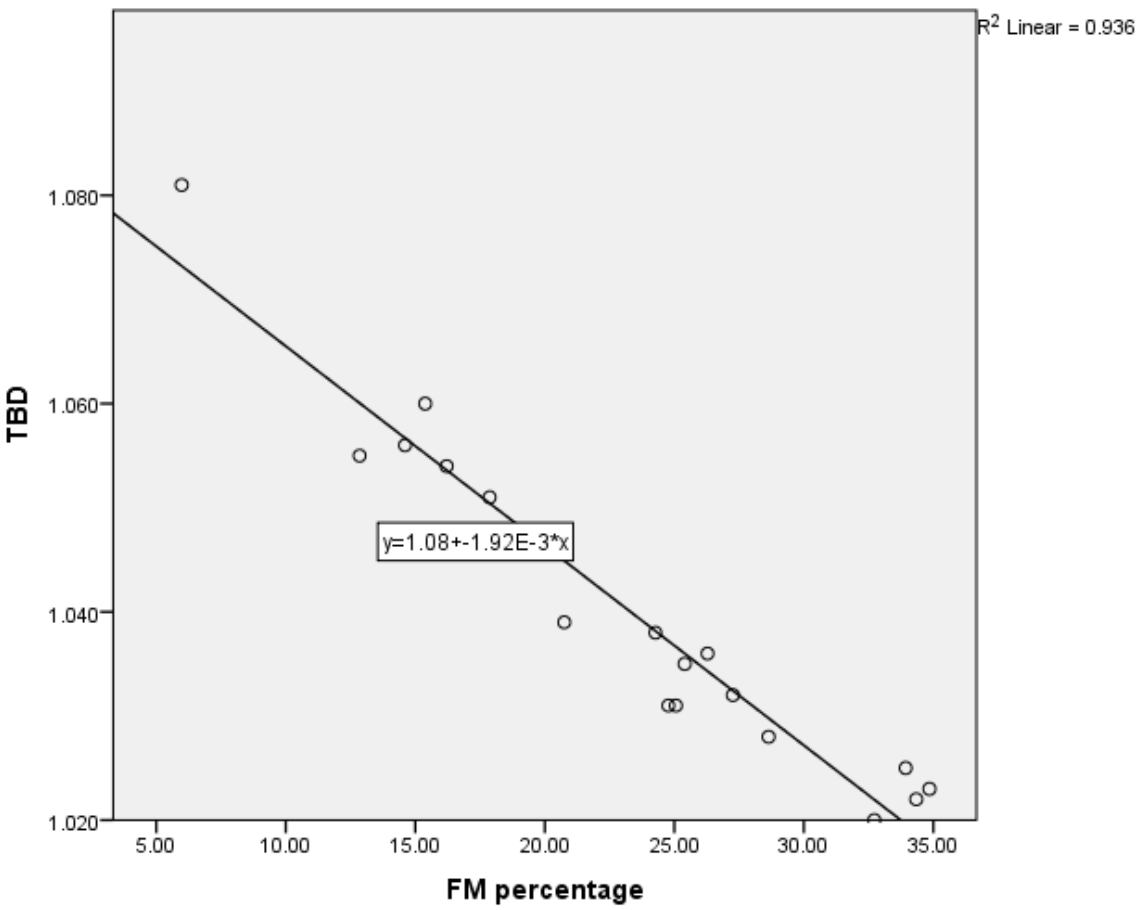

Figure 3.8: Relationship between FM% and TBD
FM fat mass, TBD total body density

As expected there was a strong negative correlation between total body density and fat mass percentage. Fat mass has a stable density of 0.9094 gr/cm^3 and fat free mass has a higher density than fat mass, therefore the more fat in the tissue the lower the density. This relationship remained strong when we looked at the participants all together, and when we looked at them separately (healthy controls and patients).

Four of the patients had BOD POD measurements post liver transplant as well. One of them, who was not transplanted, but was treated for HCV and was 1 year post sustained viral response, also had her BOD POD measurement repeated.

Table 3-23: Indices derived from BOD POD; comparison before and after liver transplant

Age	ID Sex	TpTx	BMI	BMI sds	FM	FM%	FFM	TBD	FM sds	FFM sds	TBD sds
5.16	3M		17.18	1.18	5.38	24.77	16.32	1.031	1.1	-0.15	-1.08
6.96		1.66	17.3	1.08	7.65	29.27	18.48	1.025	1.18	-0.55	-1.59
10.54	11F		19.35	0.86	8.09	24.27	25.23	1.038	-0.3	-0.58	0.11
12.24		1.45	19.53	0.49	9.17	22.14	32.23	1.045	-0.39	-0.23	0.56
17.17	15M		21.3	0.27	18.63	34.34	35.63	1.022	0.91	-3.22	-1.63
18.3		0.57	20.37	-0.35	19.47	34.07	37.68	1.022	0.86	-3.18	-1.55
8.7	17F		15.45	-0.44	8.03	32.72	16.5	1.020	0.12	-2.32	-0.89
9.6		0.56	15.24	-0.78	8.02	31.7	17.27	1.022	-0.10	-2.59	-0.77
16.69	16F		21.06	0.12	20.56	34.85	38.45	1.023	0.66	-0.88	-0.73
17.86		1.0	24.99	1.2	28.03	39.93	42.17	1.012	1.24	-0.16	-1.29

In light blue the measurements before liver transplant and in white the measurements after liver transplant. The last 2 lines are the patient with the HCV infection, before and after treatment.
Age in years, M male, F female, TpTx time from transplant, BMI body mass index, sds standard deviation score, FN fat mass, FFM fat free mass, TBD total body density

By performing a paired t-test for the 4 patients that had a liver transplant, we found no statistical significant difference between any on the indices before and after liver transplantation.

There was significant correlation between FM (r= 0.987, $p<0.05$), FFM (r=0.965, $p<0.05$), FM sd score (r=0.993. $p<0.01$) and FFM sd score (r=0.026, $p<0.05$) before and after liver transplantation. There was significant correlation between BMI (r=0.983, $p<0.05$) and BMI sd score (r=0.966, $p<0.05$) before and after liver transplantation for these 4 patients.

FM% post-transplant significantly correlated with TBD sd score before transplant (r= -0.951, $p<0.05$) and TBD post-transplant (r=-0.96, $p<0.05$). FFM post-transplant correlated with BMI before transplant (r=0.967, $p<0.05$) and FFM sd score post-transplant correlated with FM5 before transplant (r=-0.998, $p<0.01$). Overall there were no significant correlations between FM, FFM and BMI before transplant and equally none after liver transplantation.

In summary, the BODPOD could only be done by the older patients. Looking at 8 of these patients they all had a FM% of over 10% and their FM sd score was within normal limits. In regards to FFM through, 3 of them (in spite of the organomegaly) had a low FFM sd score < 1.96. From the 10 healthy children, only one had a very low FM and FFM sd score; he was the one identified by the deuterium studies with a FM < 10%. The four patients with FM and FFM sd

scores before and after liver transplant showed no significant difference in these scores after liver transplant. The one patient that had the HCV infection treated, but remained cirrhotic, had a noticeable increase in FM sd score. As expected total body density had a strong negative correlation with FM%.

3.1.5 Dual-energy X-ray absorptiometry (DXA)

According to the study protocol only patients over the age 4 years were to have a DXA scan. Eleven of the 17 patients fitted this requirement and had their whole body DXA scan. Of these 11 patients, 6 were female. Their age varied from 4.7 to 17.2 years (mean 10.69, median 9.89 and SD 4.78 years).

DXA allows for total and regional body composition data. For each patient, DXA will give values of total FM, bone free FFM (LM) and BMC. The FM and LM was expressed in sd scores to allow comparisons between the patients, using data from the reference populations previously mentioned (Wells et al., 2012, Wells et al., 2010b). FM and LM can also be expressed as fat mass index (FMI) and lean mass index (LMI) and then again in sd scores. FMI and LMI are calculated by a method analogous to BMI (FM and LM divided by Ht^2 respectively). In addition it will give values of arm, leg and trunk FM, LM, which can then be converted to the relevant indices and subsequent sd scores. This helps remove the effect of height, which is of particular relevance in children that may be stunted (Wells et al., 2002).

Table 3-24 and Table 3-25 show the sd scores for the FM and FMI measurements for the children before liver transplantation. One child had a BMI sd score below -2, but no sd scores for the FM related indices was below 2. In addition when running a paired t-test comparing these FM sd scores to 0, this population differed from 0 significantly only for the arm FM sd score (-0.70 +/- 0.66, $p<0.01$) and for the arm FMI sd score (-0.59 +/- 0.78, $p<0.05$).

Table 3-24: SD scores for FM based on DXA

ID Sex	Height sds	Weight sds	BMI sds	totalFM sds	armFM sds	legFM sds	trunkFM sds
1f	2.67	-0.02	**-2.21**	-1.36	-1.57	-1.51	-1.16
3m	0.38	1.06	1.18	0.31	-0.25	0.30	0.61
6m	-1.77	-1.66	-0.52	-0.48	-0.06	-0.12	-0.44
7f	-1.70	-0.88	0.34	-0.15	-1.13	-0.46	0.39
11f	-1.55	-0.14	0.88	-0.79	-0.85	-0.99	-0.61
13f	**-3.88**	-1.61	0.80	-0.28	-0.09	-0.61	-0.16
14m	**-2.54**	**-2.16**	-0.57	-0.45	-1.13	-0.52	-0.28
15m	**-2.32**	-1.20	0.37	0.10	0.21	0.25	-0.15
16f	0.65	0.44	0.28	0.00	-0.15	-0.15	0.13
17f	-0.88	-0.77	-0.41	-0.79	-1.05	-0.85	-0.64
18m	-0.04	-0.48	-0.61	-1.07	-1.67	-1.05	-1.01

Sds standard deviation score, BMI body mass index, FM fat mass, f female, m male

Table 3-25: SD scores for FMI scores based on DXA

ID Sex	Height sds	Weight sds	BMI sds	totalFMI sds	armFMI sds	legFMI sds	trunkFMI sds
1f	2.67	-0.02	**-2.21**	-1.68	-1.79	-1.86	-1.38
3m	0.38	1.06	1.18	0.36	-0.35	0.26	0.51
6m	-1.77	-1.66	-0.52	0.04	0.08	0.14	-0.15
7f	-1.70	-0.88	0.34	-0.13	-1.16	-0.34	0.34
11f	-1.55	-0.14	0.88	-0.39	-0.54	-0.56	-0.22
13f	**-3.88**	-1.61	0.80	0.41	0.49	0.22	0.45
14m	**-2.54**	**-2.16**	-0.57	0.04	-0.66	0.00	0.12
15m	**-2.32**	-1.20	0.37	0.39	0.39	0.60	0.25
16f	0.65	0.44	0.28	-0.11	-0.24	-0.23	0.02
17f	-0.88	-0.77	-0.41	-0.52	-0.86	-0.62	-0.36
18m	-0.04	-0.48	-0.61	-1.16	-1.82	-1.20	-0.96

Sds standard deviation score, BMI body mass index, FMI fat mass index, f female, m male

Table 3-26: Correlation between FM and FMI from the DXA

	Total FM sds	Arm FM sds	Leg FM sds	Trunk FM sds	Total FMI sds	Arm FMI sds	Leg FMI sds	Trunk FMI sds
Total FM sds	N/A	0.771**	0.935***	0.938***	0.887***	0.696*	0.869**	0.907** *
Arm FM sds	0.771**	N/A	0.813**	NS	0.824**	0.949***	0.847**	0.702*
Leg FM sds	0.935** *	0.813**	N/A	0.804**	0.849***	0.710**	0.886***	0.791**
Trunk FM sds	0.938** *	NS	0.804**	N/A	0.7768*	NS	0.724**	0.889** *
Total FMI sds	0.887** *	0.824**	0.849***	0.788**	N/A	0.877***	0.985***	0.963** *
Arm FMI sds	0.696*	0.949***	0.710*	NS	0.877***	N/A	0.888***	0.752*
Leg FMI sds	0.869**	0.847**	0.886***	0.724*	0.985***	0.888***	N/A	0.912** *
Trunk FMI sds	0.907** *	0.702*	0.791**	0.889***	0.963***	0.753**	0.912***	N/A

*p<0.05, **p<0.01, ***p<0.001, NS not significant correlation, N/A not applicable
BMI body mass index, FM fat mass, FMI fat mass index, z standard deviation score

In Table 3-26 we can see the correlations between the FM and FMI sd scores derived from DXA for the patients prior to liver transplantation. In addition to the values shown in the table, arm FM sd score also correlated with weight (r= 0.611, p< 0.05) and as expected, total FMI sd score had a negative correlation with height (r= -0.709, p<0.05), as did arm FMI sd score (r=-0.646, p<0.05), leg FMI sd score (r= -0.700, p<0.05) and trunk FMI sd score (=-0.662, p<0.05).

BMI sd score correlated strongly with all sd scores of variables: total FM (r= 0.754, p<0.01), arm FM (r=0.604, p<0.05), leg FM (r=0.619, p<0.05), trunk FM (r=0.736, p<0.05), total FMI (r=0.782, p<0.05), arm FMI (r=0.624, p<0.05), leg FMI (r= 0.719, p<0.05) and trunk FMI (0.833, p<0.01).

Table 3-27 and Table 3-28 show the LM and LMI sd scores respectively for the children before liver transplantation.

Table 3-27: SD scores for LM based on DXA

ID Sex	Height sds	Weight sds	BMI sds	totalLM sds	armLM sds	legLM sds	trunkLM sds
1f	2.67	-0.02	**-2.21**	-0.47	**-2.18**	-1.57	0.84
3m	0.38	1.06	1.18	0.54	-1.29	-0.80	**2.23**
6m	-1.77	-1.66	-0.52	**-2.53**	-0.90	**-2.32**	**-2.24**
7f	-1.70	-0.88	0.34	-1.15	-1.12	-1.94	-0.26
11f	-1.55	-0.14	0.88	-0.16	-1.71	-0.92	0.94
13f	**-3.88**	-1.61	0.80	**-2.51**	**-2.87**	**-2.78**	-1.84
14m	**-2.54**	**-2.16**	-0.57	**-2.62**	**-2.54**	**-2.61**	**-2.08**
15m	**-2.32**	-1.20	0.37	-1.88	**-4.11**	**-3.00**	-0.41
16f	0.65	0.44	0.28	0.13	-0.15	-0.28	0.67
17f	-0.88	-0.77	-0.41	-0.85	**-2.97**	**-2.43**	-0.20
18m	-0.04	-0.48	-0.61	-0.23	-0.69	-0.41	0.11

Sds standard deviation score, BMI body mass index, LM lean mass, f female, m male

Table 3-28: SD scores for LMI scores based on DXA

ID Sex	Height sds	Weight sds	BMI sds	totalLMI sds	armLMI sds	legLMI sds	trunkLMI sds
1f	2.67	-0.02	**-2.21**	**-2.34**	**-4.05**	**-3.51**	0.01
3m	0.38	1.06	1.18	1.04	-1.75	-1.21	3.92
6m	-1.77	-1.66	-0.52	-1.79	0.03	**-2.00**	-1.39
7f	-1.70	-0.88	0.34	0.28	-0.43	-1.42	1.42
11f	-1.55	-0.14	0.88	1.92	-0.89	0.33	3.30
13f	**-3.88**	-1.61	0.80	0.14	-0.98	-0.77	0.93
14m	**-2.54**	**-2.16**	-0.57	-1.52	-1.69	-1.84	-0.62
15m	**-2.32**	-1.20	0.37	-0.82	**-4.42**	**-2.53**	1.40
16f	0.65	0.44	0.28	0.06	-0.29	-0.49	0.70
17f	-0.88	-0.77	-0.41	-1.00	**-3.41**	**-2.66**	0.94
18m	-0.04	-0.48	-0.61	-0.42	-0.95	-0.62	0.10

Sds standard deviation score, BMI body mass index, LMI lean mass index, f female, m male

In contrast to the FM and FMI sd scores, many more LM and LMI sd scores are below -1.96. When this data was compared with a paired t-test to 0, total LM sd score (-1.07 +/- 1.15, $p<0.05$), arm LM sd score (-1.87 +/- 1.18, $p<0.001$), leg LM sd score (-1.73 +/- 0.99, $p<0.001$, arm LMI sd score (-1.71 +/- 1.55, $p<0.01$) and leg LMI sd score (-1.52 +/- 1.12, $p<0.01$) differed significantly from 0, whereas total LM, total LMI, trunk LM and trunk LMI did not.

Table 3-29: Correlations of LM and LMI sd scores from whole body DXA

	Total LM sds	Arm LM sds	Leg LM sds	Trunk LM sds	Total LMI sds	Arm LMI sds	Leg LMI sds	Trunk LMI sds
Total LM sds	N/A	NS	0.863***	0.951***	NS	NS	NS	NS
Arm LM sds	NS	N/A	0.787**	NS	NS	0.762**	NS	NS
Leg LM sds	0.863***	0.787**	N/A	0.715*	NS	NS	NS	NS
Trunk LM sds	0.951***	NS	0.715*	N/A	NS	NS	NS	0.736*
Total LMI sds	NS	NS	NS	NS	N/A	NS	0.825**	0.843**
Arm LMI sds	NS	0.762**	NS	NS	NS	N/A	0.735*	NS
Leg LMI sds	NS	NS	NS	NS	0.825**	0.735*	N/A	NS
Trunk LMI sds	NS	NS	NS	0.736**	0.843**	NS	NS	N/A

*correlation was statistically significant with a p<0.05, ** correlation was statistically significant with a p<0.01,
*** correlation was statistically significant with a p<0.001, NS not significant, N/A not applicable
BMI body mass index, LM lean mass, LMI lean mass index, z standard deviation score

Table 3-29 shows the correlations between the LM and LMI sd scores. In addition to the ones shown in the table, Height sd score correlated significantly with total LM sd score (r=0.744, p<0.01), leg LM sd score (r= 0.651, p<0.05) and trunk LM sd score (r=0.702, p<0.05). Weight sd score correlated significantly with total LM sd score (r=0.958, p<0.001), leg LM sd score (r=0.802, P<0.01), trunk LM sd score (r=0.959, p<0.001) and trunk LMI sd score (r=0.64, p<0.05). BMI sd score correlated significantly with the following sd scores: total LMI (r=0.853, p<0.01), leg LMI (r=0.68, p<0.05) and trunk LMI (r=0.706, p<0.05).

None of the FM or FMI sd scores, either regional or total correlated significantly with any of the LM or LMI sd scores either regional or total.

Four children had a whole body DXA before and after liver transplantation. The two children that were removed for the liver transplantation list also had repeat whole body DXA scans. In Table 3-30 and in Table 3-31 we can see the FM sd scores and the FMI sd scores before and after liver transplantation for the 4 children that had liver transplants.

Table 3-30: SD scores for FM measurements by DXA before and after liver transplantation

Age	ID Sex	Height sds	Weight sds	BMI sds	totalFM sds	armFM sds	legFM sds	trunkFM sds
Pre Tx								
5.16	3M	0.38	1.06	1.18	0.31	-0.25	0.30	0.61
10.54	11F	-1.55	-0.14	0.88	-0.79	-0.85	-0.99	-0.61
17.17	15M	**-2.32**	-1.20	0.37	0.10	0.21	0.25	-0.15
8.66	17F	-0.88	-0.77	-0.41	-0.79	-1.05	-0.85	-0.64
Post Tx								
6.91	3M	0.30	1.07	1.25	1.20	0.83	0.58	1.25
12.24	11F	-0.78	-0.02	0.48	-0.94	-0.63	-1.11	-0.91
18.42	15M	**-2.07**	-1.27	0.16	0.56	0.79	0.60	0.40
9.58	17F	-1.18	-1.15	-0.70	-0.62	-0.45	-0.62	-0.49

Age in years, Pre Tx before transplantation, Post Tx after transplantation, M male, F female, sds standard deviation score, BMI body mass index, FM fat mass

Table 3-31: SD scores for FMI measurements by DXA before and after liver transplantation

Age	ID Sex	Height sds	Weight sds	BMI sds	totalFMI sds	armFMI sds	legFMI sds	trunkFMI sds
Pre Tx								
5.16	3M	0.38	1.06	1.18	0.36	-0.35	0.26	0.51
10.54	11F	-1.55	-0.14	0.88	-0.39	-0.54	-0.56	-0.22
17.17	15M	**-2.32**	-1.20	0.37	0.39	0.39	0.60	0.25
8.66	17F	-0.88	-0.77	-0.41	-0.52	-0.86	-0.62	-0.36
Post Tx								
6.91	3M	0.30	1.07	1.25	1.11	0.87	0.67	1.20
12.24	11F	-0.78	-0.02	0.48	-0.80	-0.51	-0.91	-0.76
18.42	15M	**-2.07**	-1.27	0.16	0.83	0.96	1.00	0.66
9.58	17F	-1.18	-1.15	-0.70	-0.17	-0.18	-0.27	-0.15

Age in years, Pre Tx before transplantation, Post Tx after transplantation, M male, F female, sds standard deviation score, BMI body mass index, FMI fat mass index

When the mean difference in sd score for the FM and FMI measurements was compared between the 2 groups with a paired t-test, only the difference in arm FM z score before and after transplant achieved statistical significance at $p<0.05$.

BMI sd score before transplant correlated significantly with BMI sd score after transplant ($r= 0.975$, $p<0.05$), as did total FM sd score ($r=0.985$, $p<0.05$), Leg FM sd score ($r=0.988$, $p<0.05$) and trunk FM sd score ($r=0.963$, $p<0.05$), but not arm FM sd score. These correlations were not significant for total FMI, arm FMI, leg FMI and trunk FMI sd scores before and after liver transplant.

In Table 3-32 and Table 3-33, the LM sd scores and the LMI sd scores before and after liver transplantation for the 4 children that had liver transplants are shown. Two of these children had significantly low arm and leg LM and LMI sd scores before their liver transplant and these remained low after their liver transplant.

Table 3-32: SD scores for LM measurements by DXA before and after liver transplantation

Age	ID Sex	Height sds	Weight sds	BMI sds	totalLM sds	armLM sds	legLM sds	trunkLM sds
Pre Tx								
5.16	3M	0.38	1.06	1.18	0.54	-1.29	-0.80	2.23
10.54	11F	-1.55	-0.14	0.88	-0.16	-1.71	-0.92	0.94
17.17	15M	**-2.32**	-1.20	0.37	-1.88	**-4.11**	**-3.00**	-0.41
8.66	17F	-0.88	-0.77	-0.41	-0.85	**-2.97**	**-2.43**	-0.20
Post Tx								
6.91	3M	0.30	1.07	1.25	-0.13	-0.41	-0.49	0.58
12.24	11F	-0.78	-0.02	0.48	0.30	0.32	-0.14	0.66
18.42	15M	**-2.07**	-1.27	0.16	**-2.66**	**-3.18**	**-2.92**	**-2.05**
9.58	17F	-1.18	-1.15	-0.70	**-2.51**	**-3.29**	**-2.91**	**-2.00**

Age in years, Pre Tx before transplantation, Post Tx after transplantation, M male, F female, sds standard deviation score, BMI body mass index, LM lean mass

Table 3-33: SD scores for LMI measurements by DXA before and after liver transplantation

Age	ID Sex	Height sds	Weight sds	BMI sds	totalLMI sds	armLMI sds	legLMI sds	trunkLMI sds
Pre Tx								
5.16	3M	0.38	1.06	1.18	1.04	-1.75	-1.21	3.92
10.54	11F	-1.55	-0.14	0.88	1.92	-0.89	0.33	3.30
17.17	15M	**-2.32**	-1.20	0.37	-0.82	**-4.42**	**-2.53**	1.40
8.66	17F	-0.88	-0.77	-0.41	-1.00	**-3.41**	**-2.66**	0.94
Post Tx								
6.91	3M	0.30	1.07	1.25	-0.40	-0.57	-0.79	0.93
12.24	11F	-0.78	-0.02	0.48	1.58	1.13	0.81	2.18
18.42	15M	**-2.07**	-1.27	0.16	**-2.11**	**-3.10**	**-2.39**	-1.36
9.58	17F	-1.18	-1.15	-0.70	**-2.66**	**-3.52**	**-3.09**	-1.74

Age in years, Pre Tx before transplantation, Post Tx after transplantation, M male, F female, sds standard deviation score, BMI body mass index, LMI lean mass index

When the mean difference in sd score for the LM and LMI measurements was compared between the 2 groups with a paired t-test, there was a statistically significant difference ($p < 0.05$) between trunk LM sd score, total LMI sd score and trunk LMI sd score. All three decreased post liver transplantation.

Leg LM sd score, LMI sd score and leg LMI sd score pre transplant had a strong positive correlation with the corresponding measurements post-transplant ($r = 0.968$, 0.981 and 0.989 respectively, $p<0.05$).

There were no statistically significant correlations between the FM and FMI sd scores and the LM or LMI sd scores either before or after liver transplantation.

Two of the children were taken off the liver transplant waiting list. One was a 16 year old girl with HCV related cirrhosis. She was treated successfully for the HCV infection and she remained stable from a cirrhosis point of view. At the time of the second DXA scan she was not taking any diuretics. The second child was a child already transplanted because of biliary atresia, but with significant graft function. He was taken off the list, because it was felt, the dysfunction was improving and it was a matter of lapsed compliance with his immunosuppression. Table 3-34 and Table 3-35 show the sd scores for these 2 children at the

time they were considered for liver transplantation and 15 and 13 months later respectively. One can see that the girl has increased fat mass, whilst the boy has further reduced lean mass.

Table 3-34: FM sd scores for the 2 children removed from the liver transplant waiting list

Age	ID Sex	Height sds	Weight sds	BMI sds	total FMsds	arm FMsds	leg FMsds	trunk FMsds
16.66	16f	0.65	0.44	0.28	0.00	-0.15	-0.15	0.13
17.86	16f	0.67	1.38	1.24	1.03	1.02	0.71	1.22
9.25	14m	**-2.54**	**-2.16**	-0.57	-0.45	-1.13	-0.52	-0.28
10.44	14m	-1.51	-0.55	0.44	0.89	0.70	0.84	1.03

In light green the measurements for the girl and in light orange the measurements for the boy. Age in years, f female, m male, sds standard deviation score, BMI body mass index, FM fat mass

Table 3-35: FMI, Lm and LMI sd scores for the 2 children removed from the liver transplant list.

total FMI sds	arm FMI sds	leg FMI sds	trunk FMI sds	total LM sds	arm LM sds	leg LM sds	trunk LM sds	total LMI sds	arm LMI sds	leg LMI sds	trunk LMI sds
-0.11	-0.24	-0.23	0.02	0.13	-0.15	-0.28	0.67	0.06	-0.29	-0.49	0.70
1.03	1.03	0.66	1.18	0.08	0.84	-0.09	0.48	0.09	0.90	-0.18	0.58
0.04	-0.66	0.00	0.12	**-2.62**	**-2.54**	**-2.61**	**-2.08**	-1.52	-1.69	-1.84	-0.62
1.25	0.99	1.20	1.28	**-2.60**	**-2.69**	**-2.47**	**-2.27**	**-2.81**	**-2.54**	**-2.50**	**-2.25**

In light green the measurements for the girl and in light orange the measurements for the boy. Age in years, f female, m male, sds standard deviation score, BMI body mass index, FM fat mass

3.1.5.1 Bone mineral density (BMD).

The eleven children with ESCLD who had a whole body DXA also had their BMD measured by the machine. By convention the sd score reported for BMD is the one for the whole body without the head (TBLH). Two of these children were under the age of 5 years and there is not enough reference data to generate an sd score, but the BMD of these 2 children was less than the BMD of some of the under 5 years of age in the database.

The nine older children had a mean sd score for their BMD of -1.43 (SD 1.09, median of -0.8 and a range of -0.3 to -3.2). When compared to an sd score of 0, this was significantly less

(p<0.01). There was no significant correlation between TBD sd score and vitamin D levels of the patients.

Four of these 9 patients have had a post liver transplant whole body DXA scan. Their mean BMD sd score prior to liver transplant was -1.28 (+/- 1.14) and after the liver transplant -0.95 (+/- 1.56). There was no statistical difference detected between these two means. The correlation of BMD sd score before and after liver transplant, almost reached statistical significance at p=0.055.

The two children that had repeat whole body DXA scans, but who had not had a liver transplant showed some improvement in their BMD sd scores. One of them was the girl with HCV related cirrhosis who received treatment for the HCV infection. Her BMD sd score improved from -0.8 to -0.2 in 15 months. The other child was a boy, already transplanted for biliary atresia who was considered for a second liver transplant. His original BMD z score was -3.2 and this had improved to -2.2 13 months later, but remained considerably low.

In summary, the DXA could only be done by the older patients. Eleven patients had their DXA. All indices related to FM were within the normal range. This was not the same for the indices related to LM. Six of the eleven patients had LM sd score, either total or regional, well below the normal range. When this was corrected for height, four had regional LMI sd scores well below the normal range. For the patients were BMD could be referenced it was found to be lower than the normal population.

Four patients had measurements before and after transplant; FM remained within the normal range and LM remained low where it was low before transplant.

3.1.6 Bioelectrical Impedance (BIA)

All the patients and the healthy controls had BIA. Phase angle (PA) was significantly lower in the patients (mean 4.3) compared to the healthy controls (5.3) (two tailed *t*-test with a *p* value of 0.0115 (ΔX= -1.0918, CI 95% -1.9181 to -0.2655). There was no statistically significant difference of PA between the patients before (mean 4.5) and after transplantation (mean 4.27) (p value 0.6391, two-tailed paired t-test). There was no statistically significant difference between the healthy controls and the patients post liver transplant.

The mean PA before liver transplant (4.7 +/- 1.06) and the mean PA after liver transplant (4.07 +/- 0.76) tested with a paired t-test for 7 patients did not achieve statistical significance.

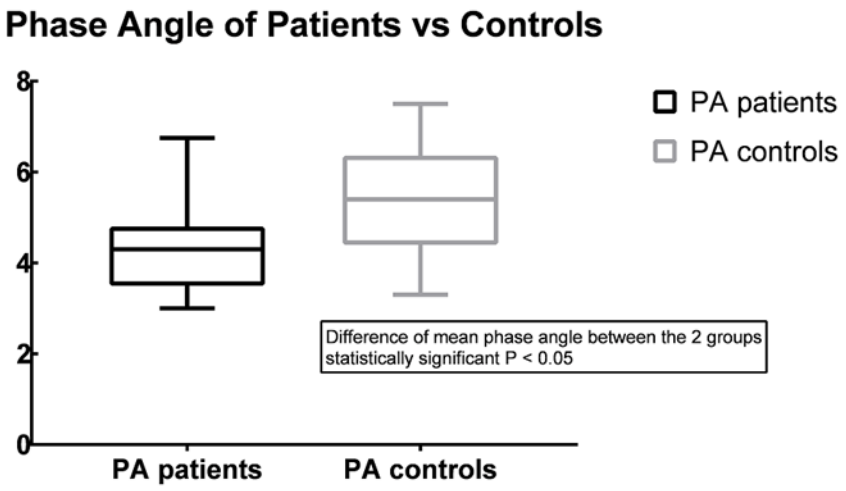

Figure 3.9 Phase angle of patients versus healthy controls
PA phase angle

Furthermore, the difference of the mean illness marker (IM) of the patients prior to liver transplantation (0.867 ± 0.057) and the mean IM of the healthy controls (0.842 ± 0.089) did not achieve statistical significance. In a similar way the mean difference of the mean IM of the patients before (0.854 ± 0.045) and after (0.851 ± 0.029) did not achieve statistical significance.

In summary, phase angle as previously described was lower in the patients in comparison to the controls, but a significant increase was not demonstrated post liver transplant. Illness marker was not significantly different between the two groups.

3.1.7 Indirect Calorimetry

3.1.7.1 REE before liver transplant

Seventeen children had their REE measured (mREE) prior to having a liver transplant with indirect calorimetry. Valid results were obtained in sixteen of them. mREE was recorded in Kcal/day and also expressed as Kcal/day/kg of body weight. In addition their predicted REE (pREE) was calculated from the Henry 2005 equations (Henry, 2005). Subsequently (REE-pREE)/pREE *100% was calculated. The children with a percentage of over +20% would be considered hypermetabolic and with a percentage of less than -20% would be considered hypometabolic. Anyone in between +20% and -20% of their predicted REE would be considered normometabolic.

Table 3-36: REE of patients before liver transplant

ID	Age (years)	Sex	Weight (kg)	mREE (kcal)	pREE (kcal)	(mREE-pREE) /pREE*100%	mREE/ kg BW
1	9.88	F	31.8	1572	1179	**33.3**	49.43
2	1.76	M	11.04	802	629	**27.5**	72.64
3	5.16	M	21.7	1166	717	**62.6**	53.73
4	0.63	F	7.14	488	408	19.6	68.35
7	4.74	F	15.45	906	803	12.8	58.64
8	0.81	M	8.08	489	441	10.9	60.52
9	3.02	F	11.4	705	719	-0.02	61.84
10	0.66	F	6.98	622	388	**60.3**	89.11
11	10.54	F	33.3	1455	1102	**32**	43.69
12	0.99	F	7.06	455	406	12.1	64.44
13	15.06	F	42.4	1646	1204	**36.7**	38.82
14	9.31	M	21.8	1284	724	**77.3**	58.90
15	17.17	M	54.26	1702	1570	8.4	31.37
16	16.7	F	59.01	1503	1434	4.8	25.47
17	8.7	F	24.53	1229	1004	18.3	50.10
18	15.7	M	53.62	1848	1599	15.6	34.46

F female, M male, kg kilograms, cm centimetres, kcal kilocalorie, mREE measured resting energy expenditure, pREE predicted resting energy expenditure, BW body weight, in bold the measurements of REE that over 20% the predicted REE.

Seven of the 16 children (44%) would qualify as hypermetabolic when using the predictive equations to compare their REE, the rest were all normometabolic 9/16 (56%) and none were hypometabolic.

Table 3-37: Comparisons between hypermetabolic and normometabolic patients

	No of patients	Mean Wt sds	Mean Ht sds	Mean BMI sds
Hypermetabolic	7	-0.63	-1.1243	0.0671
Normometabolic	9	-0.98	-1.1022	-0.3967

Wt weight, Ht height, BMI body mass index, sds standard deviation score

None of the differences of the mean z scores for weight, height or BMI between the hypermetabolic and normometabolic group achieved statistical significance using an unpaired independent t-test. Mean RQ between the 2 groups was identical at 0.77. The diagnoses of the children in the hypermetabolic group were neonatal sclerosing cholangitis, biliary atresia (3 of them), Alagille syndrome and one already transplanted for biliary atresia, being considered for a second transplant. The hypermetabolic patients in comparison to the normometabolic ones were not different in any of the liver function tests, including bilirubin levels.

In addition we measured REE for thirteen healthy children and calculated for the predicted REE from the Henry 2005 equations. Only for twelve were the results valid and can be seen below:

Table 3-38: REE of healthy controls

ID	Age (years)	Sex	Weight (kg)	mREE (kcal)	pREE (kcal)	(mREE-pREE) /pREE*100%	mREE/kg BW
C2	4.58	M	19.25	1388	678	**105**	72.10
C6	0.79	F	9.2	738	527	**40**	80.21
C5	4.5	M	17.68	1125	653	**72.3**	63.63
C7	10	M	29	1256	1106	13.6	43.31
C8	13.5	F	38.85	1560	1176	**32.6**	40.15
C9	9.67	M	32.33	1269	896	**41.6**	39.25
C10	18	F	69.14	1447	1524	-0.05	20.93
C11	15.83	M	38.76	1313	1326	-0.01	33.88
C12	4.33	F	19.78	1180	888	**32.9**	59.66
C13	6.5	F	25.15	1177	1009	16.7	46.8
C14	9.58	F	26.36	1174	1042	12.7	44.54
C15	2.67	F	18.2	949	963	-0.02	52.14

F female, M male, kg kilograms, cm centimetres, kcal kilocalorie, mREE measured resting energy expenditure, pREE predicted resting energy expenditure, BW body weight, in bold the measurements of REE that over 20% the predicted REE.

As one can see, with the previous definitions applied to the patients, 6 of the 12 (50%) healthy controls are also hypermetabolic.

Table 3-39: Comparisons between hypermetabolic and normometabolic healthy controls

	No of patients	Mean Wt sds	Mean Ht sds	Mean BMI sds
Hypermetabolic	6	0.44	0.41	0.25
Normometabolic	6	0.06	-0.06	0.06

Wt weight, Ht height, BMI body mass index, sds standard deviation score

None of the differences of the mean sd scores for weight, height or BMI between the hypermetabolic and normometabolic group achieved statistical significance using an unpaired independent t-test. The mean RQ for the hypermetabolic healthy children was 0.75 and for the normometabolic it was 0.79- this difference did not achieve statistical significance.

When comparing the two groups, patients and healthy controls, as whole groups there was no significant difference between mean age, mean mREE/kg or RQ between the two groups.

Table 3-40: Mean Age, mREE/kg and RQ between patients and healthy controls

	Age (years)	mREE/kg	RQ
Patients	7.55 (SD +6.16)	53.85 (SD +16.58)	0.77 (SD +0.036)
Controls	8.33 (SD +5.39)	49.72 (SD +16.75)	0.77 (SD +0.051)

mREE measured resting energy expenditure, kg kilograms, RQ respiratory quotient

Seven of the patients could be matched for sex and age and so for them REE and RQ was compared with a paired t-test.

Table 3-41: Mean Age, REE/kg, RQ, Weight, Height and BMI sd scores of patients and paired controls

	Mean	SD	SE mean
Age of patient	8.45	6.07	1.62
Age of control	8.27	6.03	1.61
REE/kg patient	52.03	16.83	4.50
REE/kg control	52.17	19.34	5.17
RQ patient	0.78	0.03	0.01
RQ control	0.79	0.06	0.02
Weight sd patient	-0.84	0.91	0.24
Weight sd control	-0.10	1.53	0.41
Height sd patient	-0.99	1.66	0.44
Height sd control	0.09	1.59	0.43
BMI sd patient	-0.31	0.92	0.25
BMI sd control	-0.27	1.32	0.35

SD standard deviation, SE standard error, REE/kg resting energy expenditure per kilogram, RQ respiratory quotient, sd standard deviation score, BMI body mass index

None of the above differences in mean between the various groups reached statistical significance using a paired t-test. As one can see, the mean REE/kg for the patients and their paired controls was almost identical. The mean difference between REE/kg of patient – REE/kg of paired control was -0.1621 kcal/kg (median: -0.1350 kcal/kg, min -18.37 kcal/kg and maximum +15.59 kcal/kg, SD 9.72 kcal/kg).

There was a strong negative correlation between age and REE/kg for the patients (Pearson's correlation -0.92, $p < 0.01$) and the controls (Pearson's correlation -0.938, $p<0.01$).

3.1.7.2 REE after liver transplant

For nine of the patients that have had their liver transplant we have indirect calorimetry results post liver transplant. The repeat measurements were done on average 15.6 months after the first body composition assessment (median 15.9 months, minimum 10.8 months and maximum 21.6 months) and on average 11.4 months after the liver transplant (median 10.8 months, minimum 5.2 months and maximum 20 months).

Table 3-42: Mean REE/kg and mean RQ before and after liver transplantation

	Mean	SD	SE mean
REE/kg pre	58.13	16.33	5.44
REE/kg post	58.58	18.70	6.23
RQ pre	0.77	0.04	0.01
RQ post	0.76	0.05	0.02

SD standard deviation, SE standard error, REE/kg resting energy expenditure per kilogram, RQ respiratory quotient, pre- before transplant, post- after transplant

The difference in mean REE/kg before and after liver transplantation was not statistically significant (paired t-test, p= 0.899). The difference in mean RQ before and after liver transplantation was also not significant (paired t-test, p=0.645).

REE/kg pre transplant correlated significantly with REE/kg post-transplant (Pearson's correlation factor 0.832, p= 0.005).

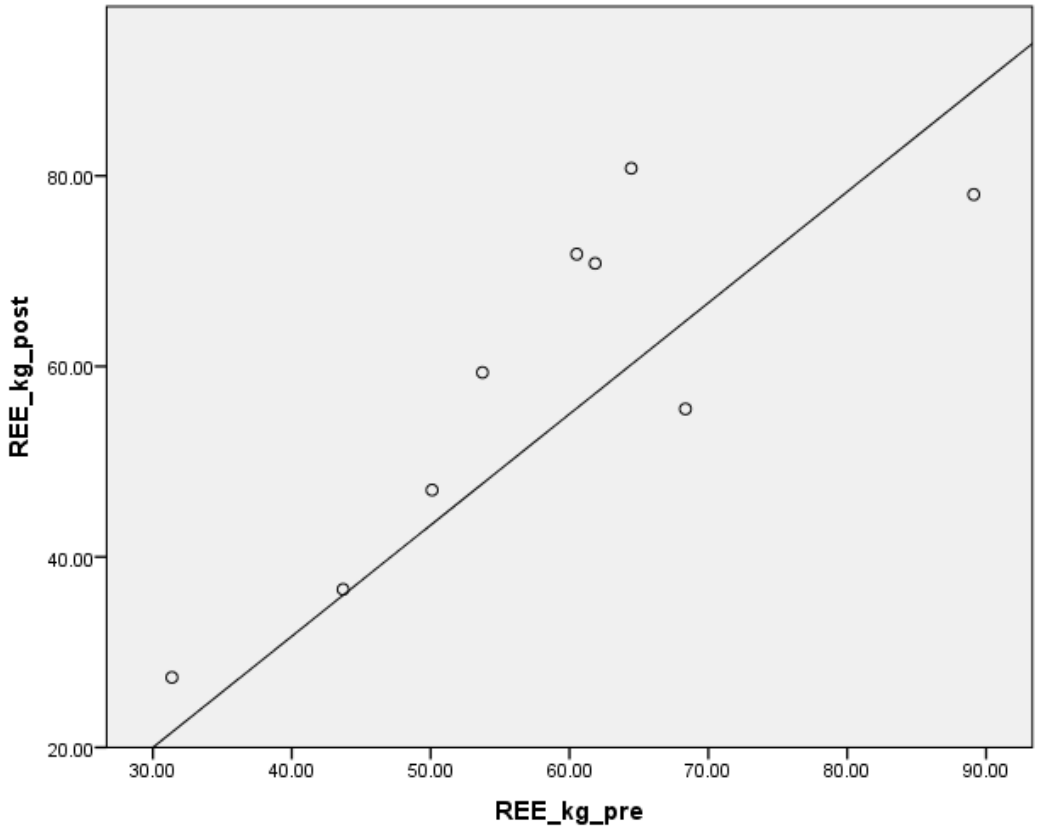

Figure 3.10: Correlation between REE/kg in kcal/kg before and after liver transplantation
REE_kg_post resting energy expenditure per kilogram of body weight after transplantation, REE_kg_pre resting energy expenditure per kilogram of body weight before transplantation

RQ before liver transplant did not correlate significantly with RQ after liver transplant.

From these 9 patients, we see that 3 of them would be classified as hypermetabolic when comparing to prediction equations. These 3 remained hypermetabolic after the liver transplant, but an additional 3 would now also classified as hypermetabolic after the transplant, making a total of 6 out of 9.

Table 3-43: Comparison of hypermetabolism before and after liver transplantation

ID Sex	Age at Transplant (years)	mREE/kg pre	mREE/kg post	% mREE over pREE pre	% mREE over pREE post
3M	5.30	53.73	59.36	62.6	95.8
4F	0.65	68.35	55.53	19.6	4.1
8M	1.09	60.52	71.78	10.9	28.9
9F	3.14	61.84	70.81	-0.02	44.5
10F	1.11	89.11	78.04	60.3	34.5
11F	10.79	43.69	36.6	32	24.8
12F	1.90	64.45	80.79	12.1	38.2
17F	9.04	50.1	47.01	18.3	16.6
15M	17.73	31.37	27.35	8.4	-3.8

mREE measured resting energy expenditure, pREE predicted resting energy expenditure, kg kilogram, pre- before liver transplant, post-after liver transplant

In summary, prediction of REE with equations underestimates REE for patients and healthy controls. The main determinant of REE/kg after transplant is the REE/kg before transplant. REE/kg reduces with increasing age.

In summary, whereas 44% of the patients were demonstrated to be hypermetabolic when using the predictive equations to compare their REE, so were 50% of the healthy children. Nine patients were measured post liver transplant and 67% were hypermetabolic. REE/kg of BW before transplant correlated strongly to REE/kg after liver transplantation.

3.1.8 3D Photonic Scanning (3D-PS)

Three of the patients had 3D photonic scanning before and after liver transplantation and the child with HCV related cirrhosis also had 3D-PS before and after treatment. Apart from the

images, 3D-PS generates anthropometric measurements and the pairs for these 4 patients are shown in the table below.

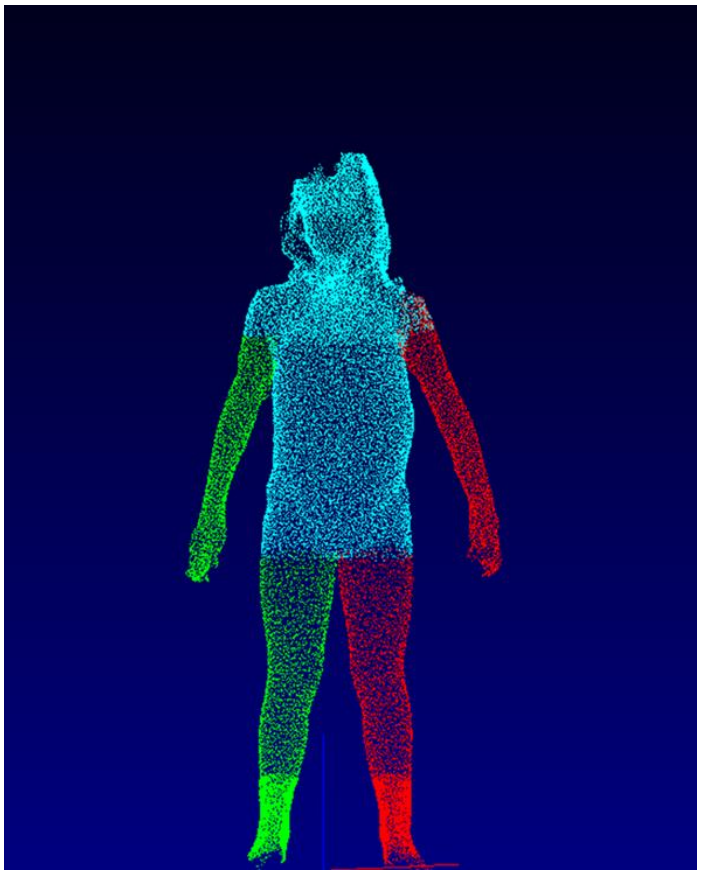

Figure 3.11: Image generated by 3D-PS of a child with ESCLD
3D-PS 3 dimensional photonic scanning, ESCLD end stage chronic liver disease

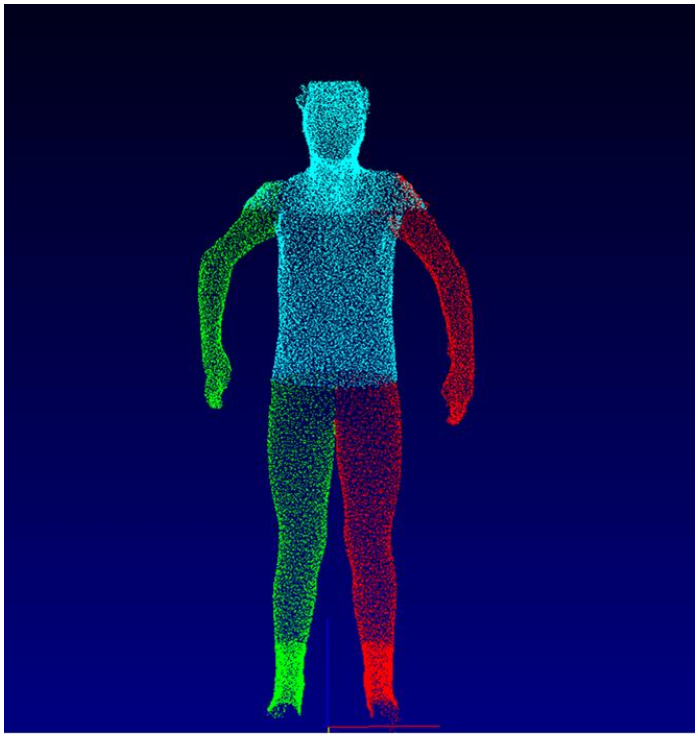

Figure 3.12: 3D-PS image of the child in Figure 3-11 after liver transplantation
3D-PS 3 dimensional photonic scanning

Table 3-44: 3D-PS measurements of children before and after liver transplantation

ID	Waist	Hips	Upper Arm	Mid-Thigh	Centre Trunk	Calf
17-pre	64.2	67.3	20.6	30.7	108.1	24.2
17-post	62.5	70.8	21.5	34.1	107.9	27.6
16-pre			25.3	44.0	155.2	34.9
16-post	93.1	103.4	33.3	54.4	145.7	37.8
11-pre	70.0	75.4	22.1	38.2	123.2	28.0
11-post	69.3	77.1	24.5	48.1	114.7	34.9
13-pre	81.3	90.0	26.3	43.4	150.1	33.6
13-post	81.9	96.5	31.0	44.5	155.5	34.5

All measurements are in centimetres. P patient, pre- before liver transplantation, post-after liver transplantation

There was a strong correlation between the thigh and the calf circumference (Pearson's correlation factor 0.963, $p< 0.001$). The hips circumference correlated with the centre trunk (0.918, $p<0.05$) and the waist (0.979, $p<0.001$). Correlations were tested with FM, FMI, LM and LMI sd scores from the whole body DXA measurements. Only waist circumference correlated with the trunk FM sd score and the trunk FMI sd score significantly (Pearson's correlation factor 0.763 with $p< 0.05$ and 0.827 with $P <0.05$ respectively). All 3 measurements though correlated significantly with total FM sd score and total FMI sd score; waist (Pearson correlation 0.93 $p<0.05$ and 0.86 $p<0.01$ respectively), hips (Pearson correlation 0.949 $p<0.01$ and 0.910 at $p<0.01$ respectively) and centre trunk circumference (0.814 $p<0.05$ and 0.741 $p<0.05$). Waist, centre trunk, thigh and calf circumference did not correlate significantly with any of the LM or LMI sd scores.

The upper arm circumference by 3D-PS correlated strongly with MUAC and also had a strong correlation with arm LM sd score (Pearson correlation 0.887 $p<0.05$) and arm LMI sd score (Pearson correlation 0.922 $p<0.001$), but did not correlate significantly with total LM or total LMI sd scores.

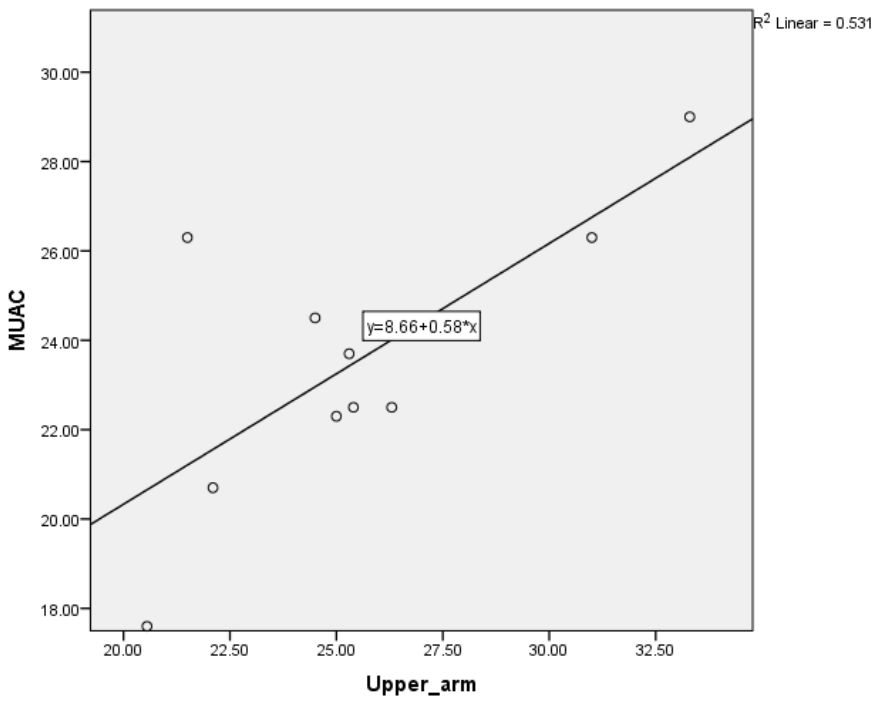

Figure 3.13: Correlation between upper arm circumference by 3D-PS and MUAC by anthropometry
3D-PS 3 dimensional photonic scanning, MUAC mid upper arm circumference, all measurements in centimetres

In summary, 3D photonic scanning was well received by the children that tried it. It is not an established method of body composition, but it did show some promising correlations with basic anthropometry and DXA derived data.

Results by 3C and 4C model of body composition

3.1.9 3C model of body composition

Eight patients and 9 healthy controls have body composition results from the 3C model, i.e. they were old enough to have the BOD POD and their deuterium samples have been analysed. Using the methodology described in the methods section, FM and FFM was calculated for each of these children and then from this the corresponding sd score.

Table 3-45: Comparison of FM and FFM of patients and healthy controls with the 3C model

	Mean	Median	SD	Range
FM sd patients	-0.06	-0.43	1.03	-1.45 to 1.97
FM sd controls	-0.094	0.06	1.26	-3.15 to 1.3
FFM sd patients	-0.85	-0.54	0.99	-2.25 to 0.45
FFM sd controls	-0.74	-1.09	1.24	-2.25 to 1.31

FM fat mass, FFM free fat mass, sd standard deviation score, SD standard deviation

The mean difference between the FM sd score of the patients and the controls and the mean difference between the FFM sd score of the patients and the controls did not achieve statistical significance. When the above sd scores were compared with an sd of zero using a paired t-test, then the only measurement that was significantly different from zero was the FFM sd score of the patients (mean difference -0.85, $p< 0.05$).

Three of the patients had results from before and after their liver transplant. The mean difference between these sd scores did not achieve statistical significance.

Table 3-46: FM and FFM before and after liver transplantation using the 3C model

	Before Transplant	After Transplant
Mean FM sd score (SD)	0.34 (± 1.41)	0.63 (± 0.36)
Mean FFM sd score (SD)	-1.32 (± 0.88)	-1.43 (± 1.11)

3C 3 component, FM fat mass, FFM free fat mass, sd standard deviation score, SD standard deviation

In summary the 3C model where applied, showed that FM sd scores were within the normal range and FFM sd scores were lower than the FM sd scores. FM and FFM sd scores did not differ significantly between the patients and the healthy controls, but the FFM sd scores did differ significantly from the normal distribution. There was no significant difference between FM and FFM sd scores for 3 patients when compared before and after liver transplant.

3.1.10 4C model of body composition

Seven patients have had a 4C model of body composition i.e. they have deuterium dilution studies (with results available), BOD POD and whole body DXA. One of these patients though had a problematic deuterium study and therefore had to be excluded from the 4C analysis. Their FM and FFM was calculated as described in the methods section and subsequently appropriate sd scores were assigned. For these measurements we do not have healthy controls to compare them, but as we have sd scores we can compare them against a value of zero with a paired t-test.

Table 3-47: FM and FFM sd scores using the 4C model

4C Model	Mean (± SD)	Median	Range
FM sd score	-0.15 (± 1.04)	-0.48	-1.53 to 1.82
FFM sd score	-1.09 (± 1.0)	-1.13	-2.24 to 0.45

4C 4 component, FM fat mass, FFM fat free mass, sd standard deviation score, SD standard deviation

When comparing with an sd of zero, only the FFM sd scores of the patients reached statistical significance (difference of the mean -1.09, $p< 0.05$).

For 2 patients with 4C body composition results expressed as sd scores, before and after liver transplantation, we can see the results in the table below.

Table 3-48: FM and FFM before and after liver transplantation using the 4C model

	Before transplant	After transplant
FM sd score (SD)	0.26 (± 1.36)	0.42 (± 0.61)
FFM sd score (SD)	-1.22 (± 0.83)	-1.38 (±1.1)

4C 4 component, FM fat mass, FFM fat free mass, sd standard deviation score, SD standard deviation

In summary, the 4C model where applied, showed that FM sd scores were within normal range and FFM sd scores were lower than the FM sd scores overall and deviated significantly from the normal distribution.

Comparison between 2C, 3C and 4C models of body composition

The 4C model can give us estimates of FM and FFM without relying on the assumptions made by the various 2C models. By comparing the individual results from DXA, BOD POD and deuterium dilution studies one can easily see, if one method has been problematic. Expression of fat mass as % can make these discrepancies more obvious.

Table 3-49: Comparison between 2C models

ID	Whole Body DXA		Deuterium		BOD POD		4CM	
	FM	%FM	FM	%FM	FM	%FM	FM	%FM
1	5.36	16.84	4.66	14.65	5.68	17.87	4.53	14.26
3	3.38	15.56	10.75	49.52	5.38	24.77	8.13	37.48
11	6.69	20.10	7.18	21.57	8.09	24.27	6.94	20.83
14	3.97	18.20	5.46	25.05	3.18	14.60	3.94	18.05
15	11.74	21.63	11.08	20.42	18.63	34.34	13.68	25.20
16	16.84	28.54	18.00	30.50	20.56	34.85	18.76	31.79
17	5.16	21.03	5.87	23.93	8.03	32.72	6.50	26.49
3p	6.09	23.31	6.55	25.05	7.65	29.27	7.05	27.00
17p	6.75	26.69	7.31	28.90	8.02	31.70	7.67	30.33

DXA dual energy absorptiometry, BOD POD air displacement plethysmography, 4CM 4 component model, FM fat mass in kilograms, %FM is percentage of fat in body weight, p post liver transplant

By plotting the average percent body fat by 2 methods against the difference of percent body fat between the same 2 methods, we can see the agreement between the 2 methods. Looking at the tables we can see that DXA and BOD POD don't have good agreement, whereas DXA does have good agreement with deuterium (aside the one outlier with the problematic deuterium study).

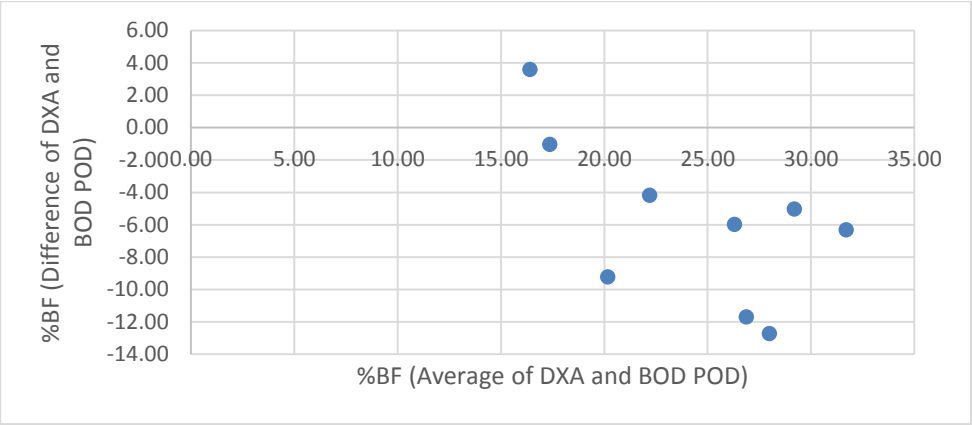

Figure 3.14: Agreement between DXA and BOD POD
DXA dual energy absorptiometry, BOD POD air displacement plethysmography, %BF percentage body fat

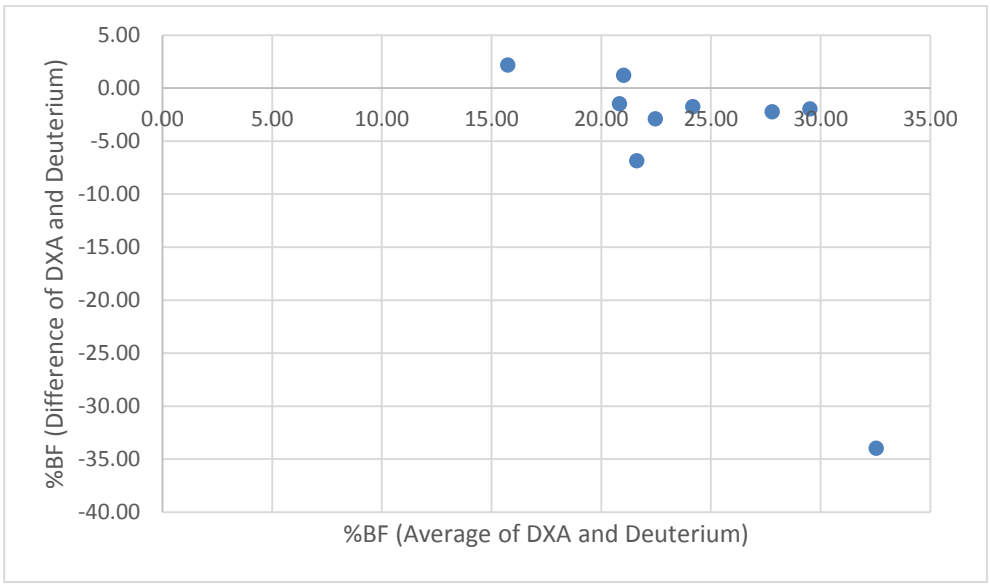

Figure 3.15: Agreement between DXA and Deuterium
DXA dual energy absorptiometry, %BF percentage body fat

Chapter 4 RESULTS- “TISSUE STUDIES”

Introduction

In this chapter are presented the results from the study of expression of metabolic related molecular pathways in the tissue of the children that had a liver transplant. Fourteen of the patients recruited have had their liver transplant and liver, muscle and fat tissue has been collected as previously described in 13 of them. The analysis presented in this thesis includes the tissue studies of 11 of these patients. The remaining 2 together with the tissue to be collected from the last patient waiting to be transplanted will be analysed as a second batch. The analysis presented in this thesis also includes tissue from 2 patients with Crigler-Najjar type 1 syndrome. Therefore in total we analysed muscle, liver and fat tissue from 13 children.

Total RNA was extracted from this tissue and amplified cDNA was made, this was then used for the microarray studies, as described in the methods section (p58-60). Two of the patient liver samples were too small to yield enough RNA and unfortunately had to be excluded from the analysis.

To summarise, 11 liver samples, 13 muscle and 13 fat tissue samples were studied. Nine of the liver samples and 11 of the muscle and 11 of the fat samples were from patients with ESCLD and 2 of the liver, muscle and fat samples were from the 2 patients with Crigler-Najjar type 1 syndrome. A list of the tissue sample IDs with the corresponding characteristics of the patient they were taken from can be seen in Table 4-1.

The microarray data was submitted to the software Qlucore Omics Explorer version 3.2. This software can represent the data by principal component analysis (PCA) plot (Figure 4.1). PCA is a “mathematical algorithm that reduces the dimensionality of the data while retaining most of the variation in the data set” (Ringner, 2008). It does this along 3 axes of maximal variability and allows in this way for the samples to be grouped in a visually meaningful way. The PCA plot shows 3 distinct clusters of tissue representing the 3 different tissues. Perhaps unsurprisingly the liver tissue shows a higher scatter, probably representing the variety of diseases affecting the liver of these patients.

Table 4-1: Characteristics of the patients corresponding to the tissue sample IDs.

Sample ID	Diagnosis	Age (years)	Sex	Ethnicity	Samples
1	NSC	10.4	f	Greek	M, F
3	BA	5.3	m	Caucasian	M, F
4	BA	0.7	f	Caucasian	L, M, F
6	Alagille	5.6	m	Caucasian	L, M, F
8	BA	1.1	m	Caucasian	L, M, F
9	A1AT	3.1	f	Caucasian	L, M, F
10	BA	1.1	f	Caucasian	L, M, F
11	BA	10.8	f	Caucasian	L, M, F
12	BA	1.9	f	Black African	L, M, F
15	PSC	17.7	m	Caucasian	L, M, F
17	BA	9	f	Caucasian	L, M, F
CN1	Crigler-Najjar	7.3	m	Middle East	L, M, F
CN2	Crigler-Najjar	5.5	m	Middle East	L, M, F

NSC neonatal sclerosing cholangitis, BA biliary atresia, A1AT alapha 1 antitrypsin deficiency, PSC primary sclerosing cholangitis, L liver, M muscle, F fat, m male, f female

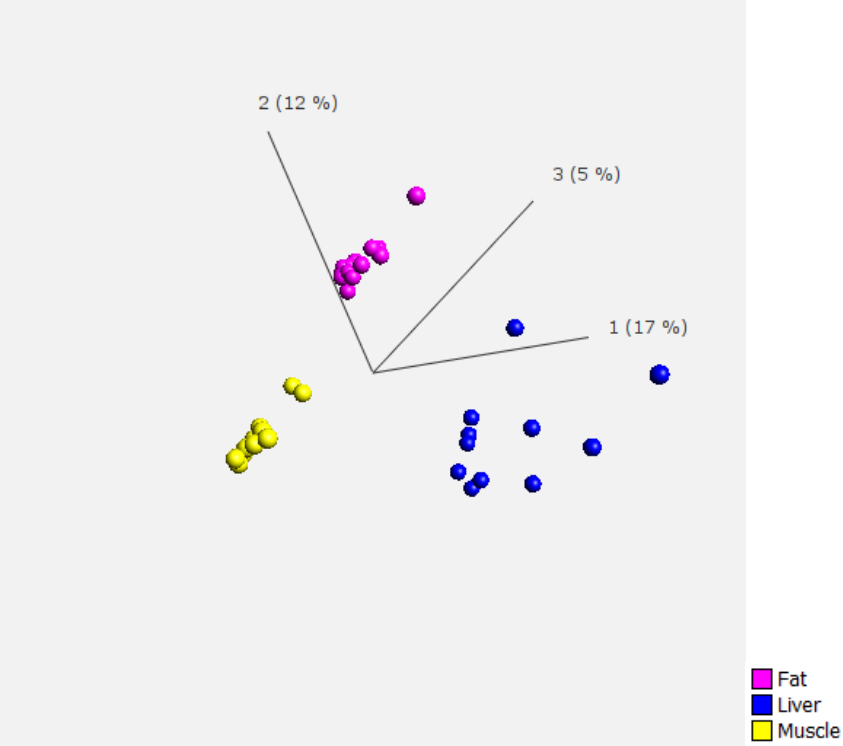

Figure 4.1: Microarray PCA plot of all samples

Muscle tissue analysis

As previously mentioned, Qlucore Omics Explorer version 3.2 software was used to detect variability between the tissue samples and the samples were studied separately for each tissue. Starting with the muscle tissue, a list of the top most variable genes was first generated. This list included 403 gene transcript IDs. The software was then asked to separate the samples according to variability, a process known as unsupervised clustering (Boutros and Okey, 2005). A q value of 0.2 and a p value of ≤ 0.05 was applied and the muscle tissue samples were separated by unsupervised clustering into 2 clusters. The first cluster included samples 3, 8, 9, 11, and 12 whereas the second cluster included samples 1, 4, 6, 10, 15, 17 and CN1 and CN2 (Figure 4.2). A two group comparison of the 2 clusters then generated a list of gene transcript IDs and their difference in expression between the 2 clusters. The “q” value in this comparison is the smallest false discovery rate for which a particular gene would remain on this list of differentially expressed genes (Storey and Tibshirani, 2003).

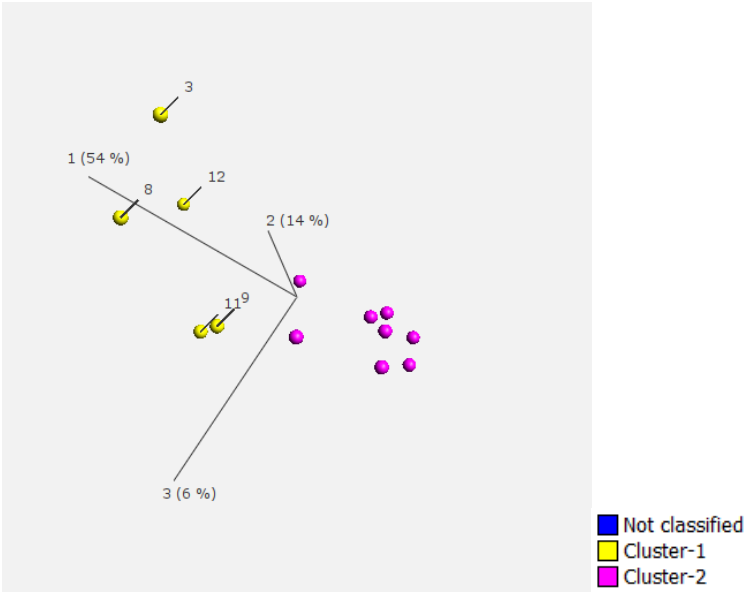

Figure 4.2: Microarray PCA plot of the 2 unsupervised clusters of the muscle samples.

Table 4-2: List of genes with a fold expression >3 between cluster 1 and cluster 2 in muscle tissue

Gene Symbol	p-value	Fold change
MT1A	0.0008	15.53
MIR21	0.0000	10.31
THBS1	0.0001	9.60
ANKRD1	0.0071	8.65
PLA2G2A	0.0010	8.17
MYH3	0.0128	6.66
MT1M	0.0003	6.55
SELE	0.0004	6.20
EGR1	0.0306	5.83
SFRP2	0.0077	5.17
EGR1	0.0306	5.83
SFRP2	0.0077	5.17
CCL2	0.0009	5.04
CH25H	0.0043	4.79
ATP1B3-AS1	0.0249	4.73
SAA1	0.0001	4.71
CTGF	0.0014	4.52
PTX3	0.0013	4.49
PRUNE2	0.0006	4.44
MYBPH	0.0007	4.40
JUNB	0.0482	4.35
PRG4	0.0292	4.25
IL6	0.0097	4.24
C13orf33	0.0005	4.22
MLLT11	0.0010	4.07
TNC	0.0070	4.07
CYR61	0.0008	4.06
ZFP36	0.0282	3.93
NAMPT	0.0008	3.82
DDN	0.0135	3.78
RGS2	0.0007	3.74
GADD45B	0.0158	3.63
CD163	0.0007	3.60
STEAP1	0.0054	3.53
SOCS3	0.0110	3.52
PRG2	0.0442	3.52
STEAP2	0.0135	3.33
RNU4ATAC	0.0447	3.26
SNORD3C	0.0469	3.24
C14orf57	0.0079	3.19
FMOD	0.0249	3.17
C7	0.0244	3.13
MYC	0.0075	3.09

F13A1	0.0064	3.08
COMP	0.0040	3.07
SCL2A3	0.0182	3.04

Table 4-3: List of genes with a fold expression <0.5 between cluster 1 and cluster 2 in muscle tissue

Gene Symbol	p-value	Fold change
KCNQ5-IT1	0.0400	0.42
COL4A3	0.0248	0.39
SIM1	0.0367	0.38
MYLK4	0.0246	0.33
DHRS7C	0.0017	0.30
KCNQ5-AS2	0.0125	0.29
MYLK3	0.0018	0.28
C3orf43	0.0088	0.28
AQP4	0.0012	0.15

In Figure 4.3, we can see a heatmap representing the muscle samples and the gene transcript IDs coloured according to fold difference of expression.

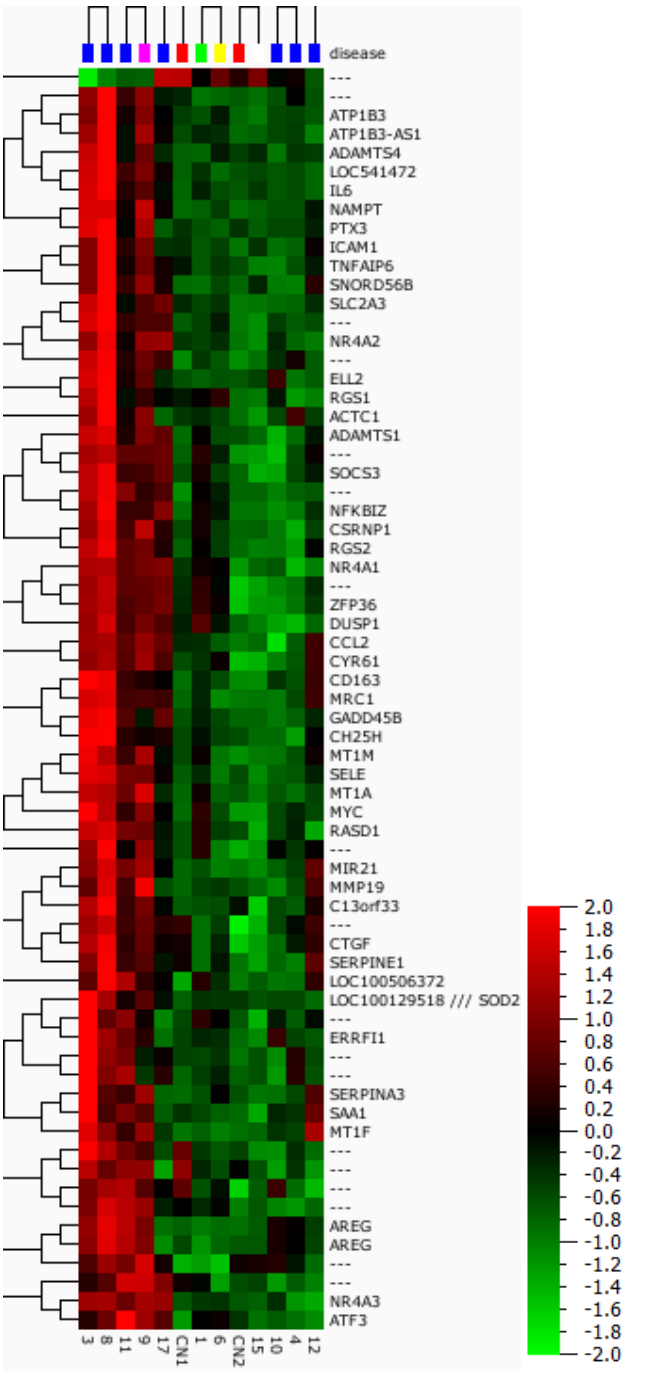

Figure 4.3: Heatmap of muscle tissue samples
Disease legend: Blue- biliary atresia, pink- a1 antitrypsin deficiency, red- Crigler-Najjar syndrome type 1, green– neonatal sclerosing cholangitis, yellow- Alagille syndrome, white- primary sclerosing cholangitis

Using GeneGo Metacore™ software we were able to analyse which molecular networks were significantly different between the two clusters. To do this we submitted the top most variable genes between the two clusters when applying cut offs of a $q \leq 0.2$ and $p \leq 0.05$ to the software. This software uses an algorithm that recognises co-regulated components of pathways or biological processed that are affected by these differentially expressed genes and then is able, based on known target gene regulation, to generate networks that include as many of the above

components possible. The 3 most significant networks generated in this way are seen in Table 4-4 and Figure 4.4 is a representation of the first pathway.

Table 4-4: Three most significant networks for cluster 1 of the muscle samples

Network	p- Value	zScore	gScore
Il-6, SAA1, SOCS3, STAT3, gp130	3.15e -06	17.88	101.63
Aggrecanase-1, collagen IV, miR-21-5p, MLCK, MHH3	4.75e -32	78	81.30
c-myc, SFRP2, FKBP11, CH25H, RPL9	1.06e -15	42.35	73.60

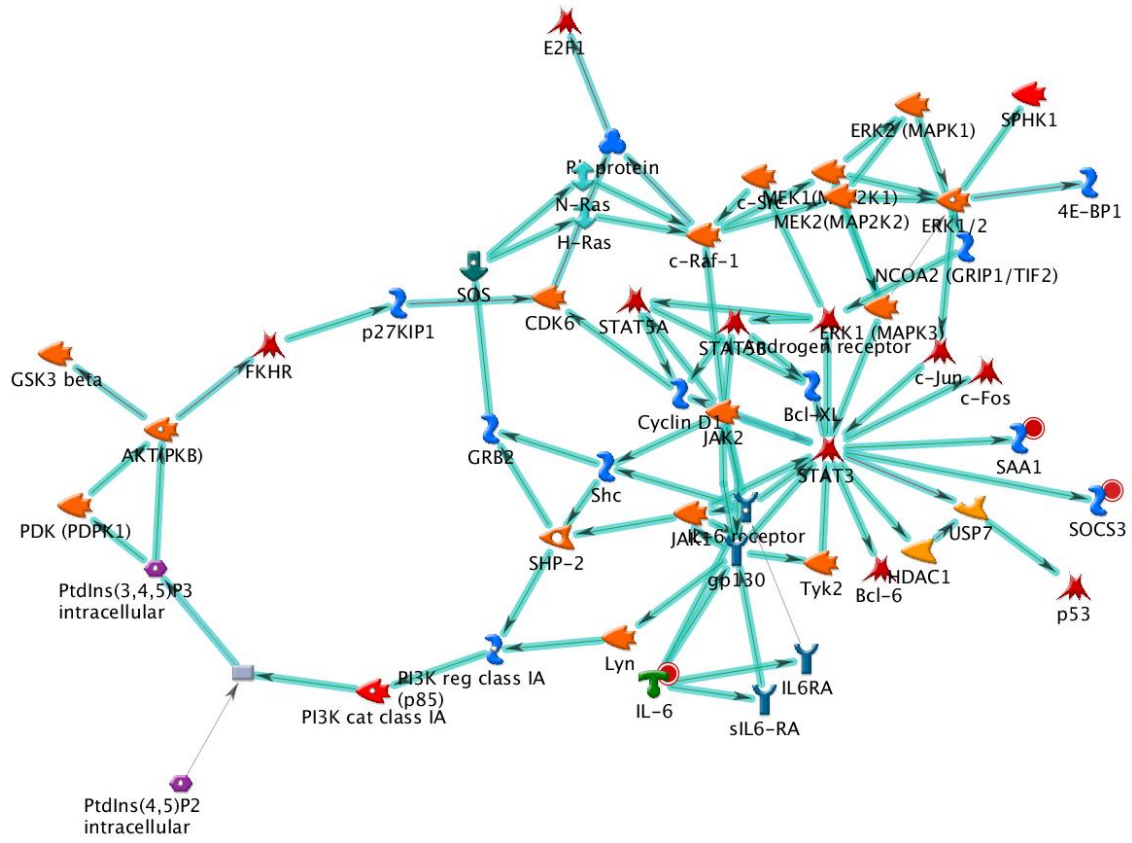

Figure 4.4: Network involving SOCS3, IL-6 and STAT3 in muscle samples of cluster 1.

Because of the interest in the AMPK and related metabolic pathways, these 2 clusters of muscle samples were also tested against a list of genes related to the AMPK pathway (see Appendix A). For this a p value ≤0.05 was used as a cut-off and the q value was not manipulated. As can be seen in Table 4-5, there was an increase in the α1 subunit of AMPK

(PRKAA1), but a reduction in PGC-1a (PPARGC1a), muscle glycogen phosphorylase (PYGM), myostatin (MSTN) and glucose transporter 4 (GLUT4- SLC2A4). Further to this in addition to the increase in IL6 and SOCS3, there was an increase in serum amyloid (SAA1), plasminogen activator inhibitor-1 (SERPINE1), as well fatty acid synthase (FAS) and transcription factor NFκB2.

Table 4-5: Top variable genes of the AMPK pathway in muscle with a p<0.05 (cluster 1 versus cluster 2)

Gene Symbol	p-value	Fold change
SAA1	0.000	4.71
IL6	0.010	4.24
RGS2	0.001	3.74
SOCS3	0.011	3.52
SERPINE1	0.001	3.43
FAS	0.008	1.43
NFKB2	0.009	1.36
STAT4	0.044	1.30
PRKAA1	0.034	1.21
SLC2A4	0.000	0.66
PYGM	0.016	0.64
PPARGC1A	0.011	0.61
MSTN	0.047	0.45

The two graphs depict the real time PCR results for the fold change in the expression of IL6 and SOCS3 confirming the microarray data.

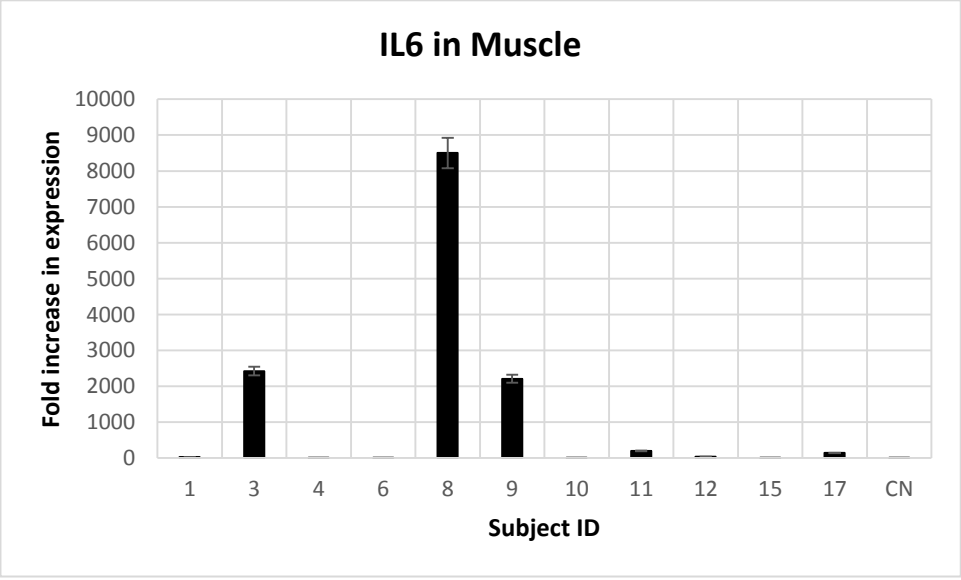

Figure 4.5: IL6 expression in muscle with PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

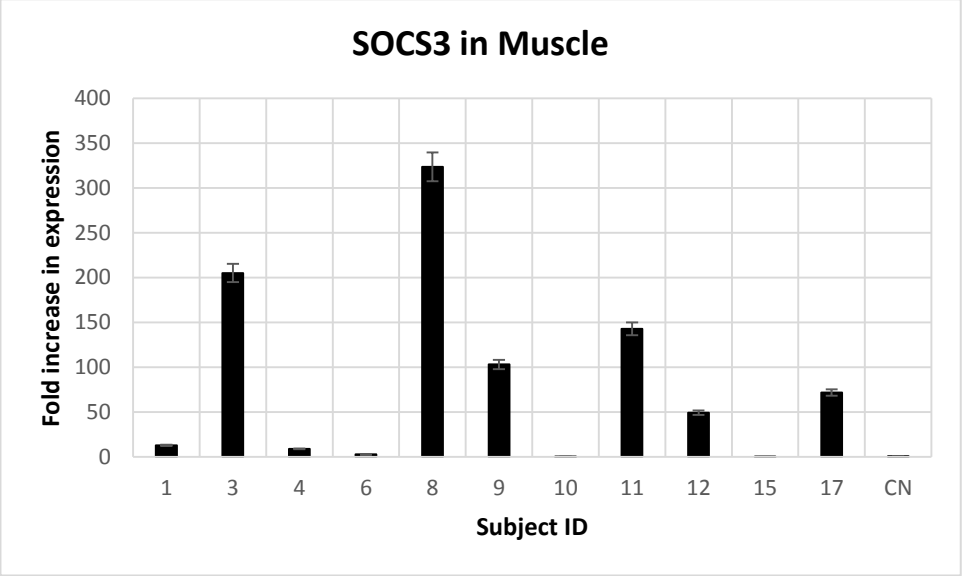

Figure 4.6: SOCS3 expression in muscle with PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

In summary, unsupervised clustering of the muscle tissue generated 2 groups with over 400 gene transcripts significantly different between the 2 groups. Based on these gene transcripts one of the most significantly different networks involved IL6- SAA1- SOCS3-STAT3-gp130, a pathway of inflammatory mediated insulin resistance, this was upregulated in group 1 (patients 3,8,9,11 and 12). The genes related to the AMPK pathway did not come up as highly variable between the 2 groups. From the evidence was for downregulation of AMPK in the muscle of group 1.

Fat tissue

Going on to analyse the fat tissue, first a list of the top most variable genes in the fat samples was generated. This list included 543 gene transcript IDs. Then by applying a q value of 0.2 and p value of ≤ 0.05 with unsupervised clustering the Qlucore Omics Explorer version 3.2 software separated the fat tissue samples into 2 clusters. The first cluster included samples 3, 8, 9, 11, and 17 whereas the second cluster included samples 1, 4, 6, 10, 12, 15 and CN1 and CN2 (Figure 4.7).

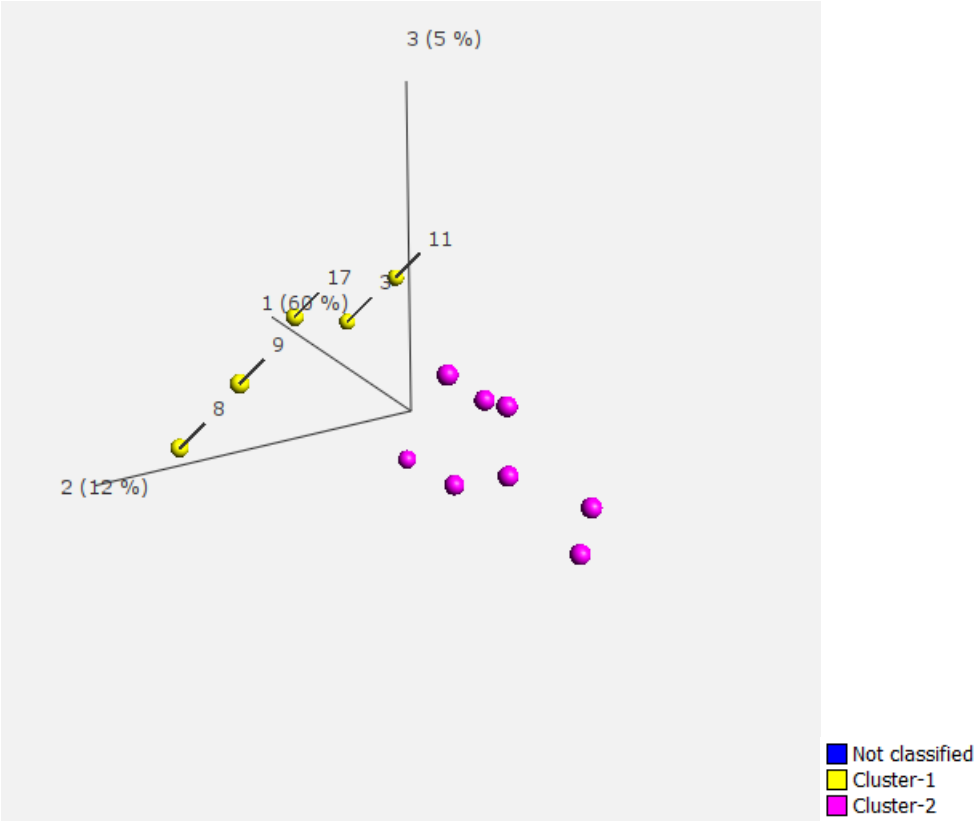

Figure 4.7: Microarray PCA plot of the 2 unsupervised clusters of the fat samples.

Table 4-6: List of genes with a fold expression >2 between cluster 1 and cluster 2 in fat tissue

Gene Symbol	p-value	Fold change
FOSB	0.0024	10.28
MT1A	0.0008	9.85
EGR1	0.0002	6.73
IL6	0.0002	6.44
SELE	0.0000	6.27
JUNB	0.0001	5.89
HSPA1A/HSPA1B	0.0006	5.39
HSPA1A /HSPA1B	0.0007	5.05
HSPA1A /HSPA1B	0.0007	5.05
HSPA1A /HSPA1B	0.0007	5.05
HSPA1A /HSPA1B	0.0008	4.98
DNAJB1	0.0008	4.96
HSPA1A /HSPA1B	0.0009	4.90
CYR61	0.0000	4.88
FOSB	0.0083	4.84
FOS	0.0214	4.80
SNORD14E	0.0150	4.79
NR4A3	0.0002	4.70
SOCS3	0.0001	4.55
HSPH1	0.0021	4.48
ATP1B3-AS1	0.0000	4.45
HSPA1A /HSPA1B	0.0010	4.36
HSPA1A /HSPA1B	0.0010	4.32
HSPA1A /HSPA1B	0.0010	4.32
MT1E	0.0021	4.10
FABP9	0.0003	4.10
TNFAIP3	0.0025	4.04
NAMPT	0.0026	4.01
HSPA1A /HSPA1B	0.0010	3.95
RNY4P20	0.0061	3.89
CCL2	0.0022	3.81
AREG	0.0334	3.81
NFKBIZ	0.0002	3.60
AREG	0.0369	3.52
TNFAIP6	0.0205	3.49
ZFP36	0.0003	3.44
CH25H	0.0345	3.42
GADD45B	0.0078	3.38
PPBP	0.0026	3.27
S100A9	0.0165	3.18
DUSP1	0.0044	3.17
NR4A2	0.0017	3.07
NR4A1	0.0030	3.05

OR52N1	0.0293	3.03
MIR21	0.0404	2.94
SERPINE1	0.0303	2.80
RNU5E-1	0.0050	2.78
IL8	0.0197	2.75
S100A8	0.0083	2.70

Using GeneGo Metacore™ software we were able to analyse which molecular networks were significantly different between the two clusters. To do this we submitted the top most variable genes between the two clusters when applying cut offs of a $q \leq 0.2$ and $p \leq 0.05$ to the software.

The 3 most significant networks are seen in Table 4-7.

Table 4-7: Three most significant networks for cluster 1 of the fat samples

Network	**p- Value**	**zScore**	**gScore**
Calgranulin B, A20, FosB, IKBZ, HSPA1B	1.66e -38	110.29	111.54
EGR1, c-Fos, ZFP36(Tristetraprolin), HSPA1A, AP-1	4.34e -23	70.85	70.85
NOR1, CCL2, Metallothionein-I, IL-8, IL-6	2.41e -20	64.27	68.02

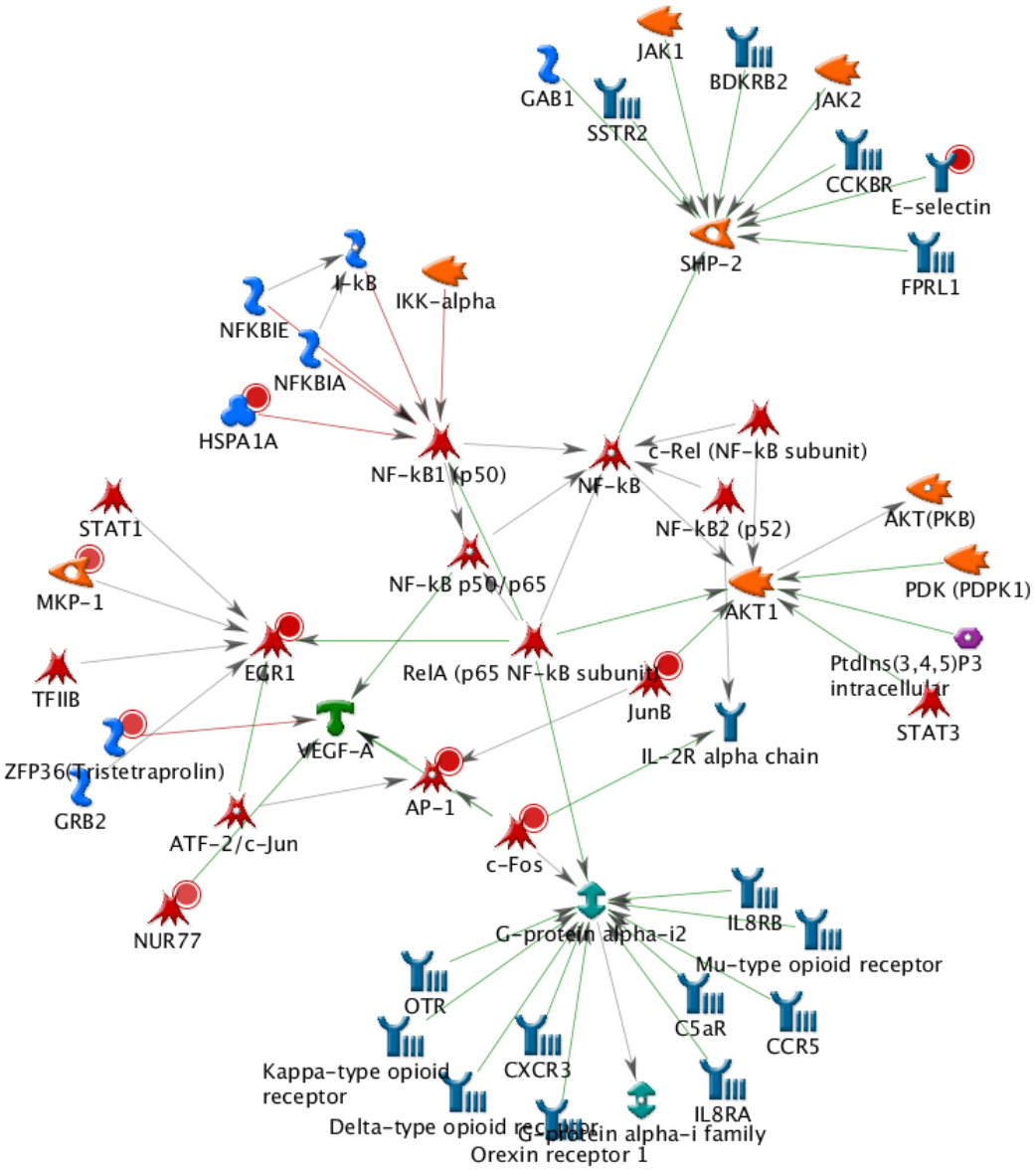

Figure 4.8: The second most significant network in the fat tissue involving AP-1.

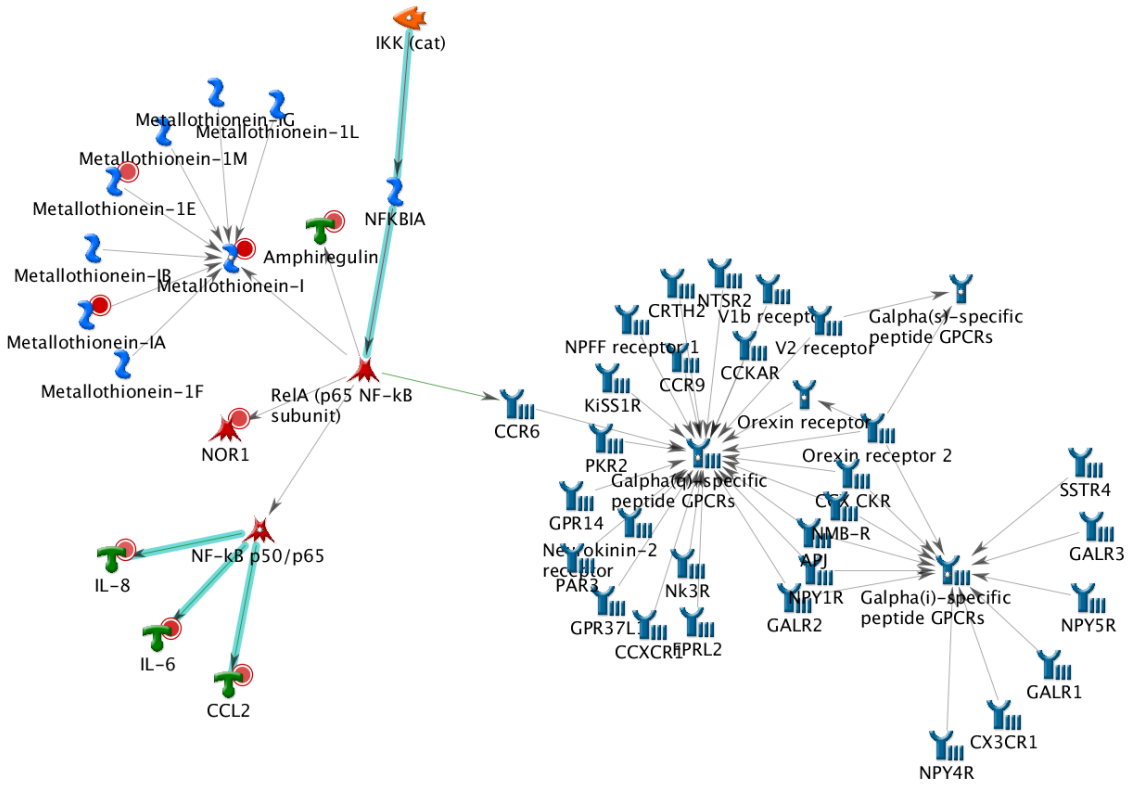

Figure 4.9: The third most significant network from the fat tissue involving IL6, IL8 and CCL2

The following three graphs depict the real-time PCR results for the fold change in the expression of IL6, SOCS3 and IL8 in the fat tissue confirming the microarray data.

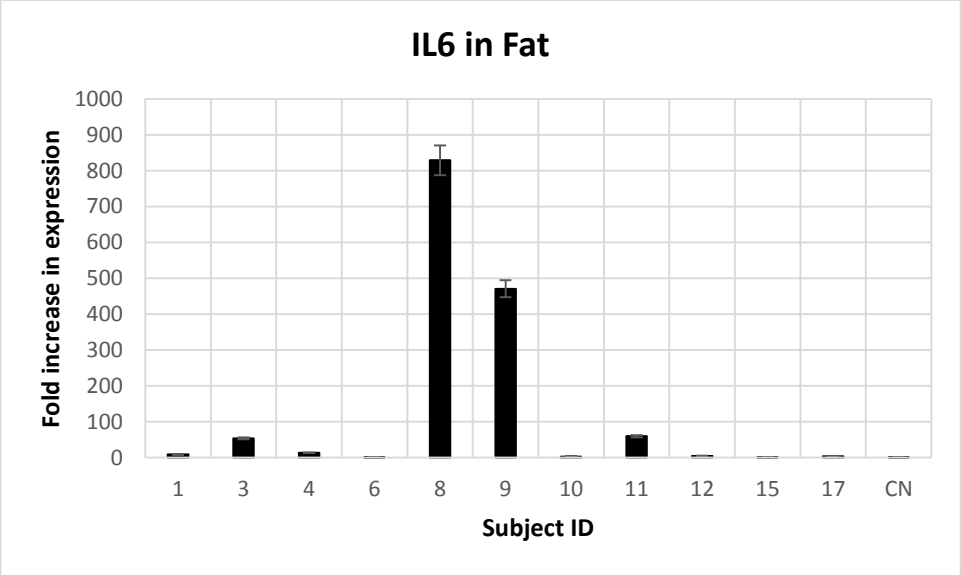

Figure 4.10: IL6 expression in fat tissue with PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

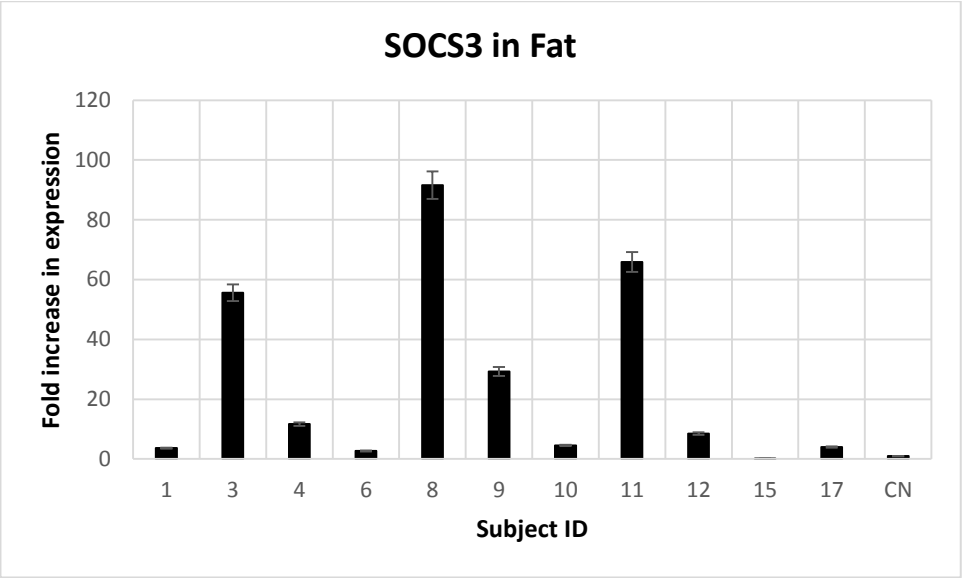

Figure 4.11: SOCS3 expression in fat with PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

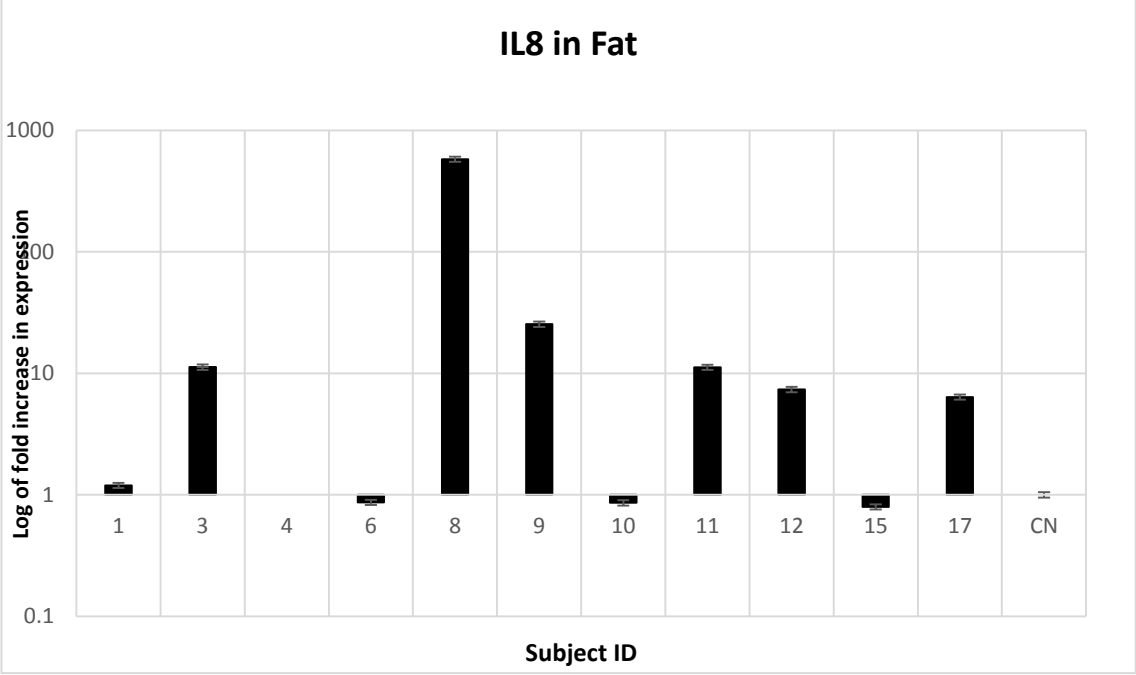

Figure 4.12: IL8 expression in fat with PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

In summary, unsupervised clustering of the fat tissue generated 2 groups with over 500 gene transcripts significantly different between the 2 groups. Based on these gene transcripts one of the most significantly different networks involved an inflammatory mediated transduction signal

pathway involving IL6-IL8-CCL2 via NFkB, which was upregulated in group 1 (patients 3,8,9,11 and 17).

4.1.1 Liver tissue

A similar process was followed for the liver tissue. First a list of the top most variable genes in the liver samples was generated with Qlucore Omics Explorer version 3.2 software. In these samples the variability was much bigger (compared to the muscle or fat samples), so even more stringent cut offs were applied. This list included 675 gene transcript IDs. By applying a q value of 0.1 and p value of ≤ 0.002 with unsupervised clustering the software separated the liver tissue samples into 2 clusters. The first cluster included samples 4, 8, 9, 10, 11, 12, 15 and 17 whereas the second cluster included samples 6, CN1 and CN2 (Figure 4.13).

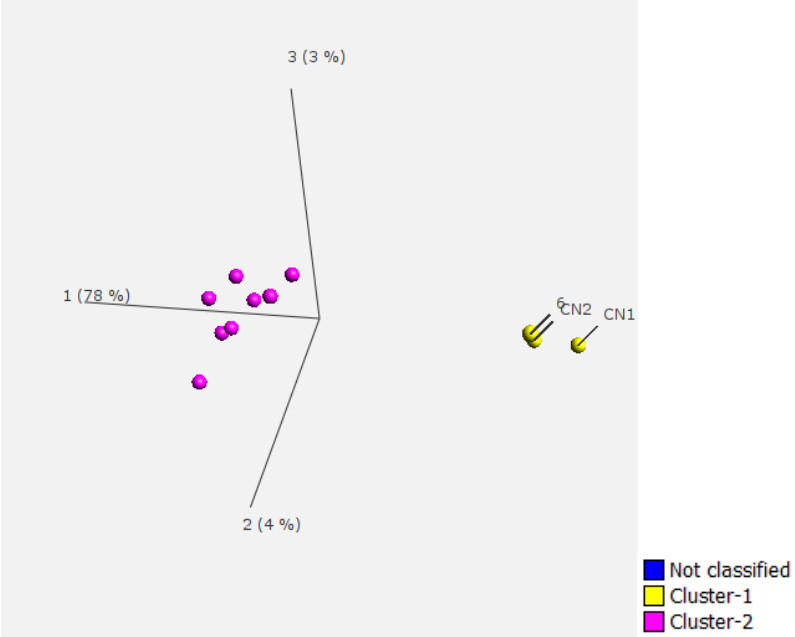

Figure 4.13: PCA plot in liver tissue- unsupervised division into 2 clusters

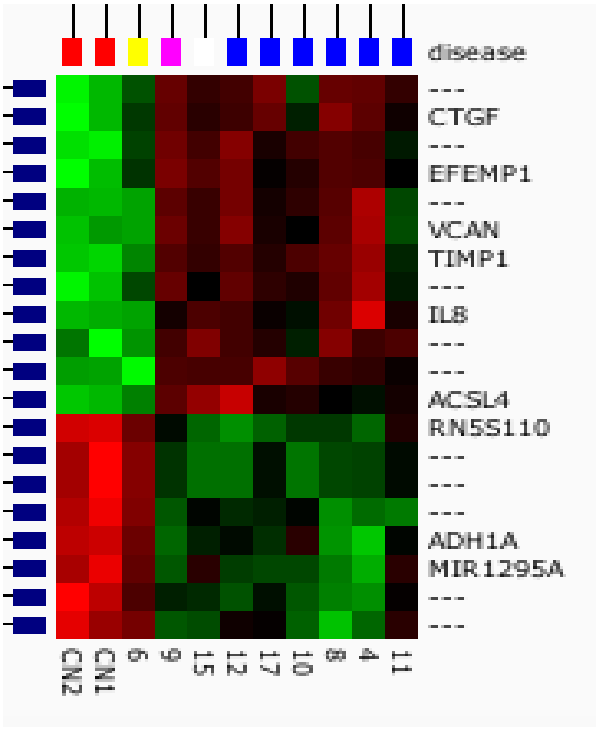

Figure 4.14: Heatmap of liver tissue showing only gene transcripts with a fold change of more than 4.
Disease legend: Blue- biliary atresia, pink- a1 antitrypsin deficiency, red- Crigler-Najjar syndrome type 1, green– neonatal sclerosing cholangitis, yellow- Alagille syndrome, white- primary sclerosing cholangitis

Table 4-8: List of known genes with a fold increase of over 4 in the liver between the 2 groups

Gene Symbol	p-value	Fold change
IL8	0.0005	9.78
VCAN	0.0009	8.90
ACSL4	0.0007	7.91
TIMP1	0.0001	5.34
CTGF	0.0007	5.08
EFEMP1	0.0006	4.88

GeneGo Metacore™ software analysed which molecular networks were significantly different between the two clusters. For this process the top most variable genes between the two clusters when applying cut offs of a $q \leq 0.1$ and $p \leq 0.05$ were submitted to the software.

Table 4-9: Three most significant networks for cluster 1 of the liver tissue

Network	p- Value	zScore	gScore
Cyclophilin C, DOC1, MUNC18-3, EIF5A2, CHMP4B	9.6e -45	58.39	58.39
PIGT, XPR1, ELTD1, TMEM32, WBP5	6.9e -40	53.28	53.28
Kif2a, Cathepsin C, Beta-adaptin 2, N4BP1, Sac2	2.9e -36	50.82	52.07

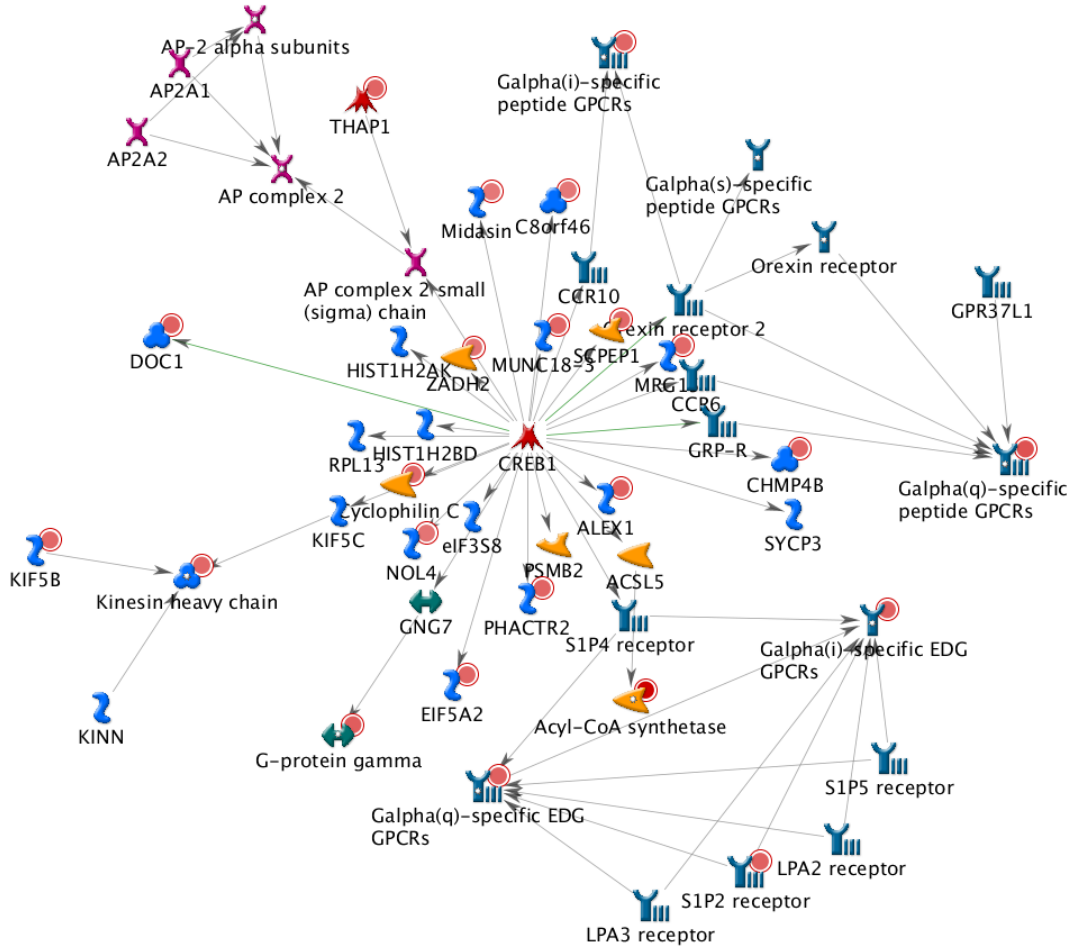

Figure 4.15: The first most significant network in the liver tissue involving CREB1

Table 4-10: Top variable genes of the AMPK pathway in liver with a p<0.05 (cluster 1 versus cluster 2)

Gene Symbol	p-value	Fold change
IL8	0.0005	9.78
SOCS3	0.0020	3.33
PFKFB3	0.0125	2.48
SERPINE1	0.0363	2.42
SAA1	0.0009	2.23
PRKAA2	0.0022	1.76
STAT3	0.0141	1.62
NFKB1	0.0079	1.40
SMAD2	0.0318	1.35
ATG13	0.0232	1.33
GYS1	0.0114	0.70
MSTN	0.0316	0.59
PRKAB2	0.0438	0.56
IL6R	0.0404	0.50

In summary, the variation of gene expression in the liver was obviously more varied than in the muscle and fat tissue, as that is the primarily diseased tissue in children with ESCLD. For this reason, unsupervised clustering for the liver tissue was applied with more stringent cut offs to the muscle and fat tissue. As perhaps expected the gene transcripts that were significantly increased in group 1 versus group 2 (group 2 included patients 6 and the 2 CN patients) were related to inflammation and fibrosis. The expression of genes related to the AMPK pathway was not in the top most variable list and there was no clear evidence to support up- or down-regulation of the AMPK pathway in the liver.

Supervised clustering

It became clear from the previous analysis that samples from subjects 3, 8, 9 and 11 were most likely different from the rest, as far as their muscle and fat tissue was concerned. This observation was tested by going back to Qlucore Omics Explorer version 3.2 and doing a supervised clustering i.e. by asking the software to treat subjects 3, 8, 9, 11 as cluster 1 and the rest as cluster 2. By setting the q value to 0.2, these 2 groups differed in 222 gene transcript IDs, all with a p value < 0.0082.

When this list was submitted to GeneGo the 3 most significant networks were generated by the software (Table 4-11).

Table 4-11: Three most significant networks for cluster 1 of the muscle samples, where cluster 1 is supervised to include subjects 3, 8, 9 and 11.

Networks	p- Value	zScore	gScore
IL-6, c-Myc, CCL2, p21, SOCS3	4.68e -19	43.27	194.52
A20, Pim-1, CCL2, MafF, Aggrecanase-1	3.02e -52	102.27	103.52
ETS2, NURR1, KLF6, MKP-1, CCL2	7.38e -32	66.76	68.01

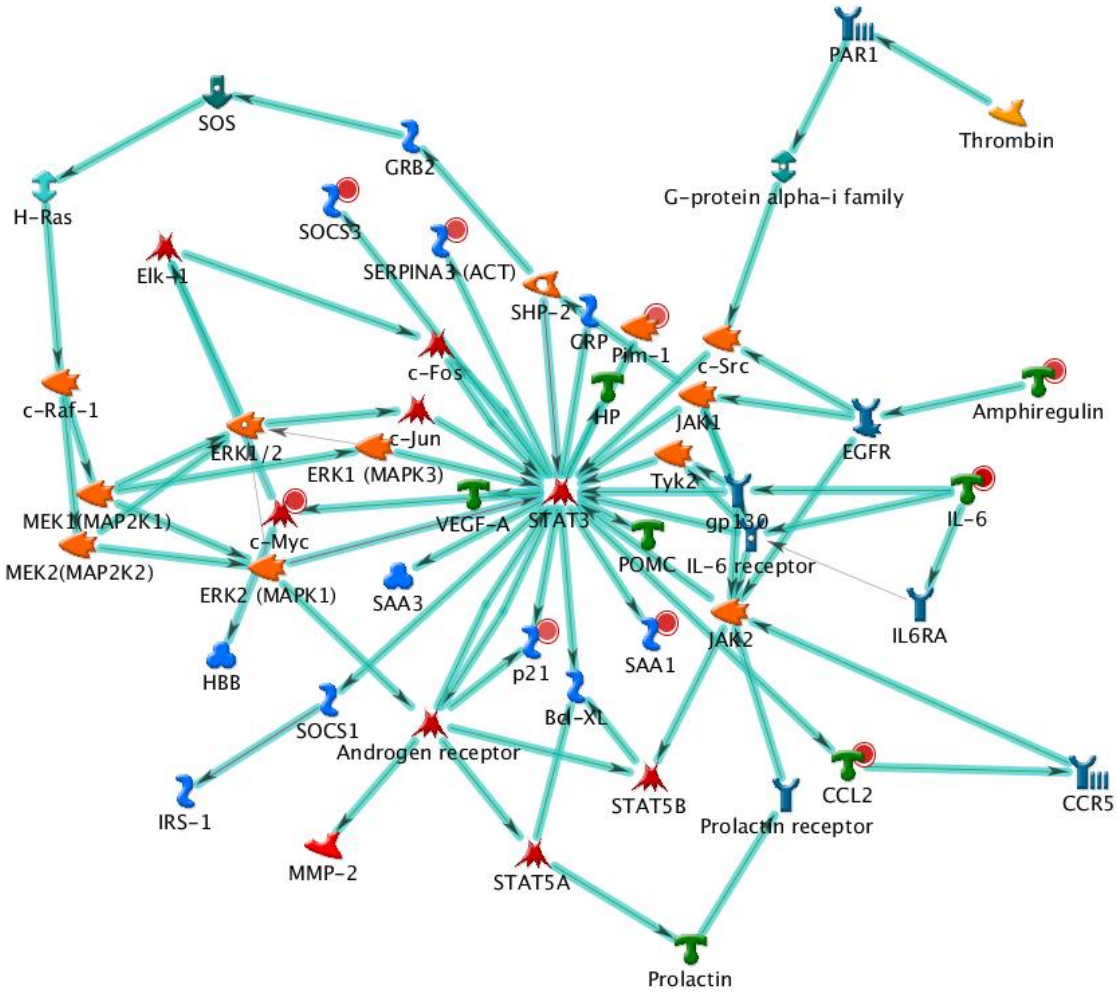

Figure 4.16The first most significant network in the muscle involving IL-6, STAT3 and SOCS3.

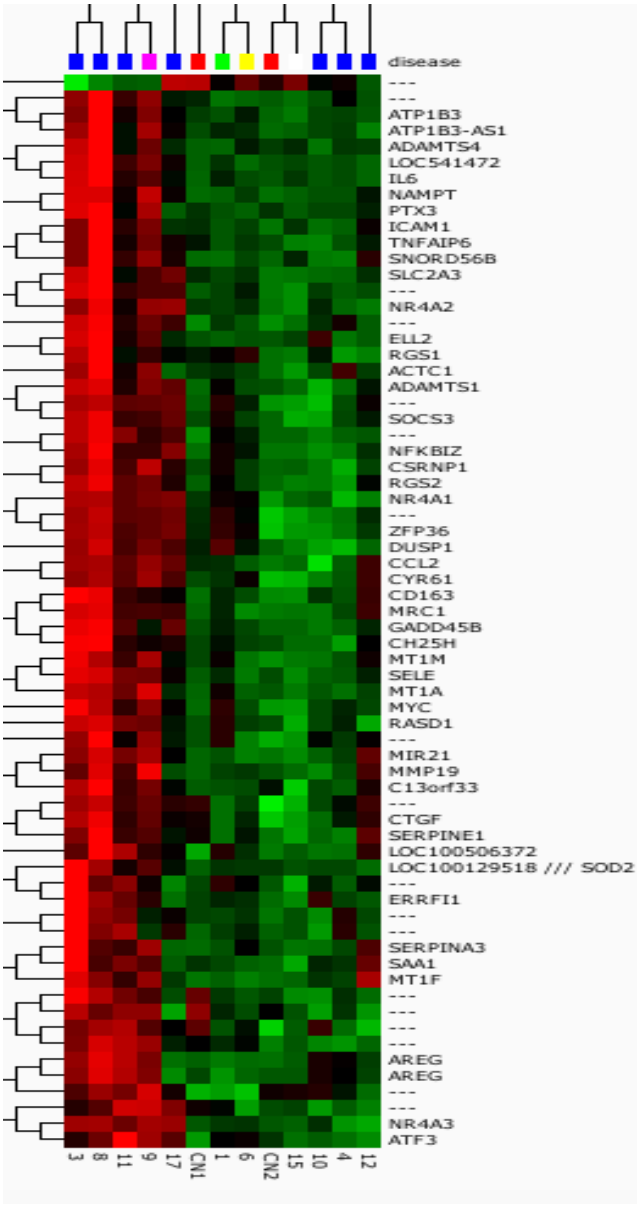

Figure 4.17: Heatmap of muscle samples after supervised clustering focusing on gene transcripts with a fold change ≥ 3.
Disease legend: Blue- biliary atresia, pink- a1 antitrypsin deficiency, red- Crigler-Najjar syndrome type 1, green– neonatal sclerosing cholangitis, yellow- Alagille syndrome, white- primary sclerosing cholangitis

Table 4-12: List of gene transcripts from the muscle tissue of subjects 3,8,9 and 11 against the rest of the muscle tissue with a fold change ≥ 3.

Gene Symbol	p-value	q-value	Fold change
MT1A	0.0000	0.01	29.27
MIR21	0.0002	0.04	9.42
ATP1B3-AS1	0.0005	0.06	8.94
SELE	0.0000	0.01	8.42
MT1M	0.0001	0.04	7.70
IL6	0.0001	0.04	6.79
PTX3	0.0001	0.04	5.77
CH25H	0.0021	0.12	5.66
ZFP36	0.0061	0.17	5.47
CCL2	0.0022	0.12	5.06
NR4A3	0.0010	0.08	4.96
RASD1	0.0001	0.04	4.89
CTGF	0.0023	0.12	4.70
C13orf33	0.0004	0.06	4.66
GADD45B	0.0040	0.14	4.65
NAMPT	0.0001	0.04	4.64
RGS2	0.0001	0.04	4.37
SOCS3	0.0034	0.13	4.32
SAA1	0.0018	0.11	4.29
ERRFI1	0.0005	0.06	4.16
MYC	0.0003	0.06	4.15
CYR61	0.0020	0.12	4.08
SLC2A3	0.0037	0.14	3.88
DUSP1	0.0033	0.13	3.84
AREG	0.0000	0.01	3.83
ADAMTS4	0.0006	0.07	3.79
ATP1B3	0.0005	0.06	3.72
CD163	0.0016	0.10	3.67
NR4A1	0.0025	0.12	3.65
SNORD56B	0.0004	0.06	3.60
NR4A2	0.0036	0.14	3.54
ACTC1	0.0007	0.07	3.52
AREG	0.0000	0.01	3.47
MMP19	0.0002	0.04	3.46
NFKBIZ	0.0054	0.17	3.41
CSRNP1	0.0001	0.04	3.37
TNFAIP6	0.0011	0.08	3.32
ADAMTS1	0.0014	0.10	3.27
ELL2	0.0005	0.06	3.25
SERPINE1	0.0033	0.13	3.25
ATF3	0.0021	0.12	3.10
MRC1	0.0013	0.09	3.07
SERPINA3	0.0009	0.08	3.07
RGS1	0.0070	0.18	3.06
ICAM1	0.0003	0.06	3.06
MT1F	0.0024	0.12	3.06

Table 4-13: Top variable genes of the AMPK pathway in muscle with a p<0.05 (supervised cluster 1 versus cluster 2)

Gene Symbol	p-value	Fold change
IL6	0.0001	6.79
RGS2	0.0001	4.37
SOCS3	0.0034	4.32
SAA1	0.0018	4.29
SERPINE1	0.0033	3.25
RGS1	0.0070	3.06
STAT4	0.0244	1.35
NFKB2	0.0168	1.35
SREBF1	0.0271	0.70
SLC2A4	0.0004	0.67

The same process was repeated with the fat tissue. The fat samples were analysed with Qlucore after submitting samples 3, 8, 9 and 11 of the fat tissue as cluster 1 and the other samples as cluster 2. This gave a list of 134 gene transcript IDs significantly different between the 2 groups at a q value of 0.2 and a p value < 0.03.

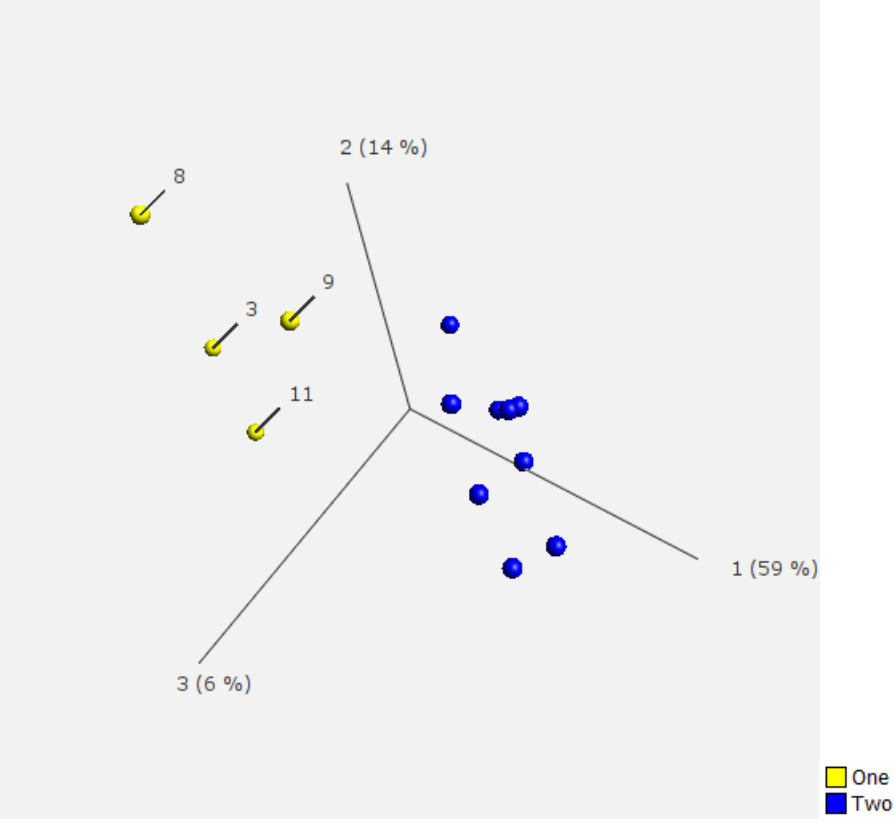

Figure 4.18: PCA of supervised clustering of fat tissue, where cluster 1 includes subjects 3, 8, 9 and 11.

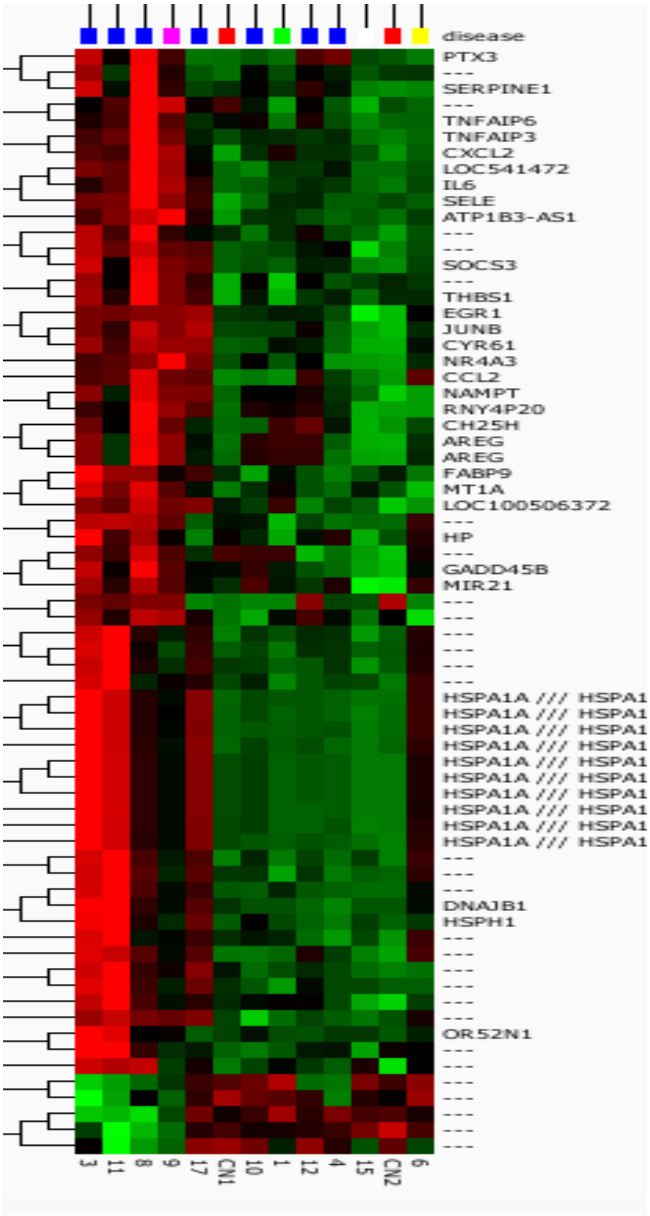

Figure 4.19: Heatmap of fat samples after supervised clustering.
Disease legend: Blue- biliary atresia, pink- a1 antitrypsin deficiency, red- Crigler-Najjar syndrome type 1, green– neonatal sclerosing cholangitis, yellow- Alagille syndrome, white- primary sclerosing cholangitis

Table 4-14: List of genes in fat tissue expressed >2.5 fold in supervised cluster 1 in comparison to cluster 2

Gene Symbol	p-value	Fold change
MT1A	0.0001	13.56
PTX3	0.0070	9.51
IL6	0.0005	6.75
SELE	0.0001	6.47
HP	0.0066	5.23
TNFAIP3	0.0003	5.13
EGR1	0.0091	5.05
ATP1B3-AS1	0.0001	4.81
AREG	0.0152	4.76
DNAJB1	0.0039	4.68
TNFAIP6	0.0049	4.56
HSPA1A /HSPA1B	0.0092	4.43
OR52N1	0.0024	4.40
CH25H	0.0125	4.36
AREG	0.0194	4.27
JUNB	0.0100	4.27
SOCS3	0.0013	4.23
HSPA1A /HSPA1B	0.0104	4.17
HSPA1A /HSPA1B	0.0103	4.14
FABP9	0.0011	4.10
HSPA1A /HSPA1B	0.0114	4.07
NR4A3	0.0040	4.06
CYR61	0.0032	4.03
HSPH1	0.0118	3.98
GADD45B	0.0037	3.90
SERPINE1	0.0056	3.67
HSPA1A /HSPA1B	0.0134	3.62
HSPA1A /HSPA1B	0.0128	3.61
HSPA1A /HSPA1B	0.0132	3.60
RNY4P20	0.0207	3.55
CCL2	0.0103	3.50
MIR21	0.0236	3.43
NAMPT	0.0205	3.39
HSPA1A /HSPA1B	0.0144	3.28
CXCL2	0.0006	3.26
THBS1	0.0014	3.18
MT1M	0.0017	3.00
NFKBIZ	0.0109	2.91
ZFP36	0.0107	2.87
IL8	0.0221	2.86
IL1RL1	0.0033	2.77
PDLIM3	0.0036	2.74
PMP2	0.0120	2.74
HSPA6	0.0026	2.72

MYC	0.0093	2.70
NFIL3	0.0015	2.64
ADAMTS4	0.0092	2.63
UBC	0.0028	2.59
MT1G	0.0050	2.58
BAG3	0.0043	2.57
ICAM1	0.0113	2.56
C5AR1	0.0176	2.55
SLC19A2	0.0050	2.53
CTGF	0.0215	2.50

The list was submitted to GeneGO Metacore™ and this generated the 3 most significant networks, shown below.

Table 4-15: Three most significant networks for supervised cluster 1 in the fat tissue

Network	**p- Value**	**zScore**	**gScore**
PAI1, VMP1, p53, Shc, GAB1	2.3e -06	18.85	165.1
ICAM1, ZFP36(Tristetraprolin), IL-6, JunB, Ubiquitin	2.76e -39	99.81	99.81
HSP70, HSC70, HSP40, IL-8, E4BP4	3.32e -33	86.48	87.73

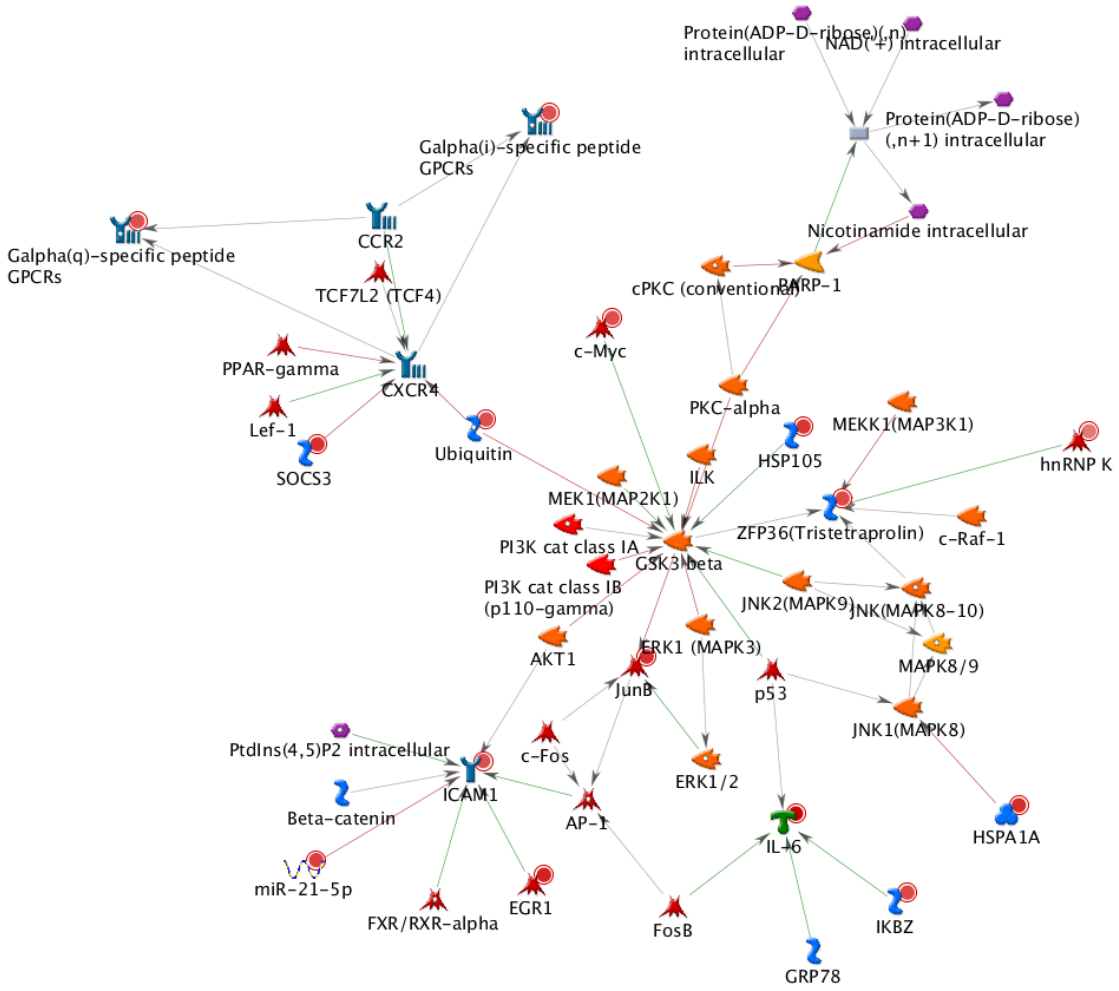

Figure 4.20: Second most significant network in the fat tissue involving IL6

Table 4-16: Top variable genes of the AMPK pathway in fat with a p<0.05 (supervised cluster 1 versus cluster 2)

Gene Symbol	p-value	Fold change
IL6	0.0005	6.75
SOCS3	0.0013	4.23
SERPINE1	0.0056	3.67
IL8	0.0221	2.86
RGS2	0.0021	2.32
CAMKK2	0.0051	0.79
IRS1	0.0240	0.70

In summary, unsupervised clustering in the muscle tissue separated subjects 3,8,9,11 and 12 to everyone else and subjects 3,8,9,11 and 17 in the fat tissue to everyone else. The clear overlap of these 2 groups was the group of subjects 3,8,9 and 11. When the group of subjects 3,8,9 and 11 was defined as a separate group in the muscle tissue (supervised clustering), as

expected, the results were similar to the unsupervised clustering highlighting again the significance of the pathway related to IL6- SOCS3 and the upregulations of various chemokines and cytokines aside IL6, like selectin E, SAA1 and CCL2. In terms of the AMPK pathway, GLUT 4 (SLC2A4) was downregulated, indirectly indication that AMPK was not activated.

When the group of subjects 3,8,9 and 11 was separated in the fat tissue again as before pathways involving IL6 and IL8 were significantly upregulated. The AMPK was not demonstrated to be different between the 2 groups of subjects. Because GLUT4 was downregulated it is upregulated by AMPK) as was CAMKK2 (it upregulates AMPK), one could infer that it was downregulated in the group of subjects 3,8,9 and 11.

4.1.2 Liver tissue

If we separate our liver samples in a supervised way and cluster 1 includes samples 8, 9 and 11 (there was no sample 3 liver tissue) the only gene trascripts that the two groups are significantly different by with a q of 0.2 are FOS and FOSB.

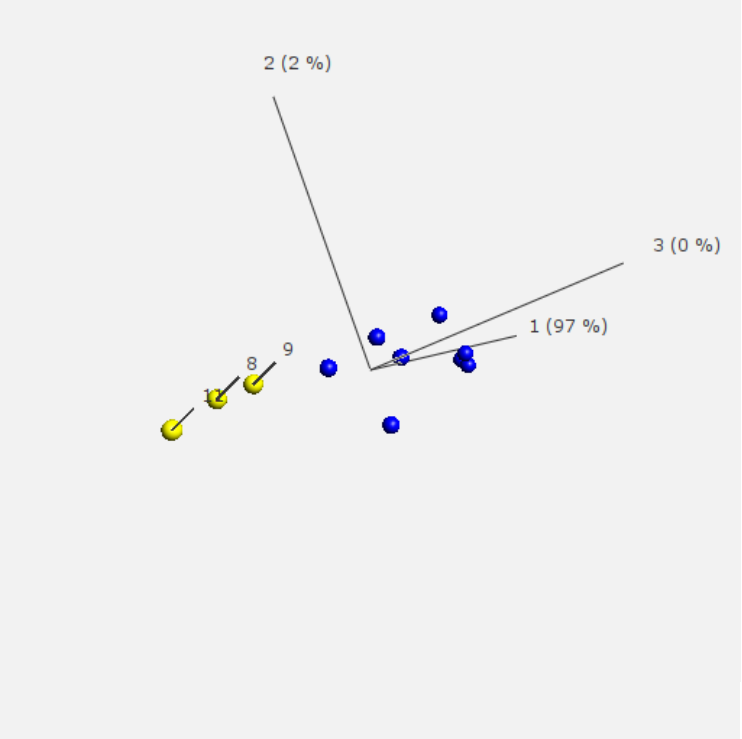

Figure 4.21: Supervised clustering of liver tissue. Cluster 1 includes subjects 8, 9 and 11

Table 4-17: The three most significant gene transcripts for liver tissue between supervised cluster 1 and cluster 2

Gene Symbol	p-value	Fold change
FOSB	0.0006	11.04
FOSB	0.0006	5.77
FOS	0.002	4.71

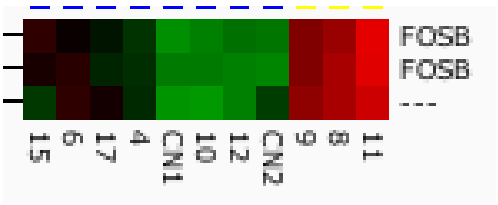

Figure 4.22: Heatmap of liver tissue with supervised clustering- subjects 8, 9 and 11 versus the rest.
Disease legend: Yellow- cluster 1, subjects 8, 9 and 11; Blue- cluster 2, subjects 4, 6, 10, 12, 15, 17, CN1 and CN2

As these children had diseases affecting the liver we also looked into a comparison of the liver tissues of the children with biliary atresia versus the 2 children with Crigler-Najjar type 1 syndrome. At a q value of 0.2 and a p value <0.05 the software generated a list of 274 gene transcript IDs. In the figure below we see a heatmap focusing on those with an over 10 fold difference in expression.

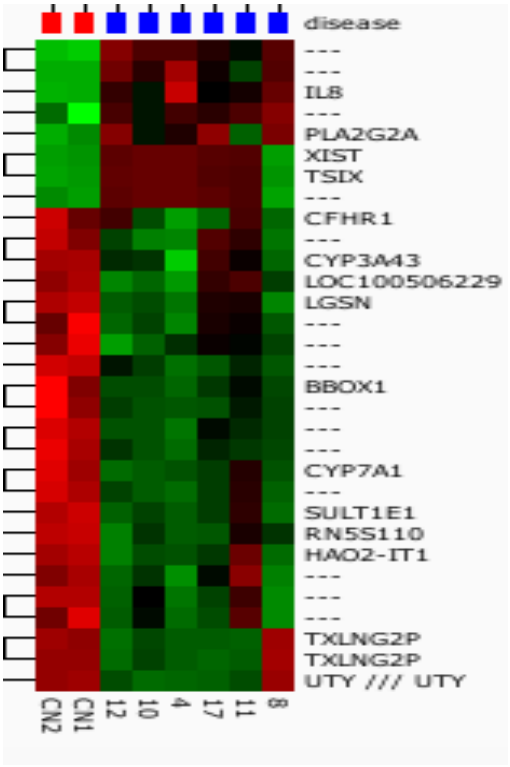

Figure 4.23: Heatmap of liver tissue with tissue from the children with biliary atresia versus the children with Crigler-Najjar type 1 syndrome. The focus is on the genes with an over 10 fold increased expression in the children with biliary atresia.
Disease legend: Red- Crigler- Najjar type 1 syndrome, Blue- biliary atresia

Table 4-18: List of gene transcripts with an over 4 fold expression in the liver tissue of children with biliary atresia when compared to those with Crigler-Najjar type 1.

Gene Symbol	p-value	Fold change
TSIX	0.0280	34.41
PLA2G2A	0.0288	17.68
IL8	0.0085	10.79
VCAN	0.0154	8.66
ACSL4	0.0061	8.07
CCL2	0.0137	7.89
CTGF	0.0011	7.44
SGIP1	0.0014	7.33
EFEMP1	0.0004	6.73
RN5S387	0.0098	6.70
TIMP1	0.0011	6.36
RASEF	0.0331	6.32
ACSM3	0.0345	6.14
MGP	0.0092	5.60
MOXD1	0.0099	5.41
BICC1	0.0297	5.18
CCDC80	0.0057	5.16
FGF7	0.0336	5.12
CCL20	0.0192	5.05
FAT1	0.0023	5.01
SULF1	0.0178	4.77
ANKRD1	0.0300	4.74
BTG2	0.0073	4.72
FAP	0.0278	4.71
RGCC	0.0098	4.63
LUM	0.0060	4.60
COL4A1	0.0251	4.57
RAB31	0.0012	4.57
ANXA1	0.0013	4.53

Table 4-19: Three most significant networks in biliary atresia versus Crigler-Najjar syndrome type 1.

Network	p- Value	zScore	gScore
G-protein alpha-i family, PAR1, SMCY, ABC50, ppl2/A4	3.79e -43	69.32	71.82
MMP-2, Fibrillin 1, SMCY, SART1, NTF2	5.42e -34	59.35	60.60
TMPS6, PRKX, MFGE8, CCL20, MGP	1.39e -33	57.95	57.95

Table 4-20: Top variable genes of the AMPK pathway in liver with a p<0.05 (biliary atresia versus Crigler-Najjar type 1)

Gene Symbol	p-value	Fold change
IL8	0.0085	10.79
PFKFB3	0.0090	3.36
SOCS3	0.0107	3.11
SAA1	0.0084	1.86
PRKAA2	0.0268	1.56
SMAD2	0.0102	1.54
NFKB2	0.0331	1.37
NFKB1	0.0469	1.25
PRKAB2	0.0370	0.43
IGF1	0.0162	0.25

The following three graphs depict real-time PCR results for the fold change in expression of IL8, and SOCS3 confirming the microarray data.

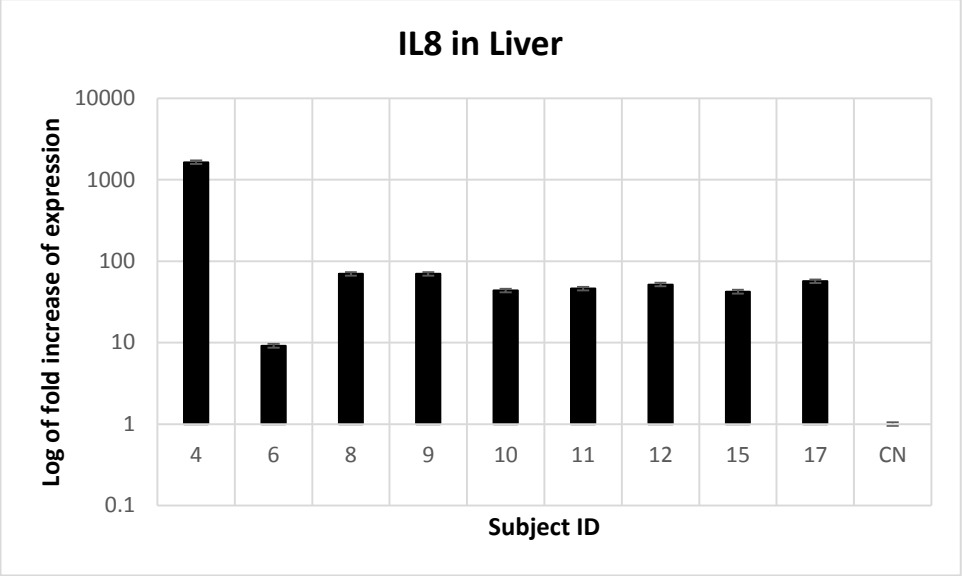

Figure 4.24: IL8 expression in the liver by PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

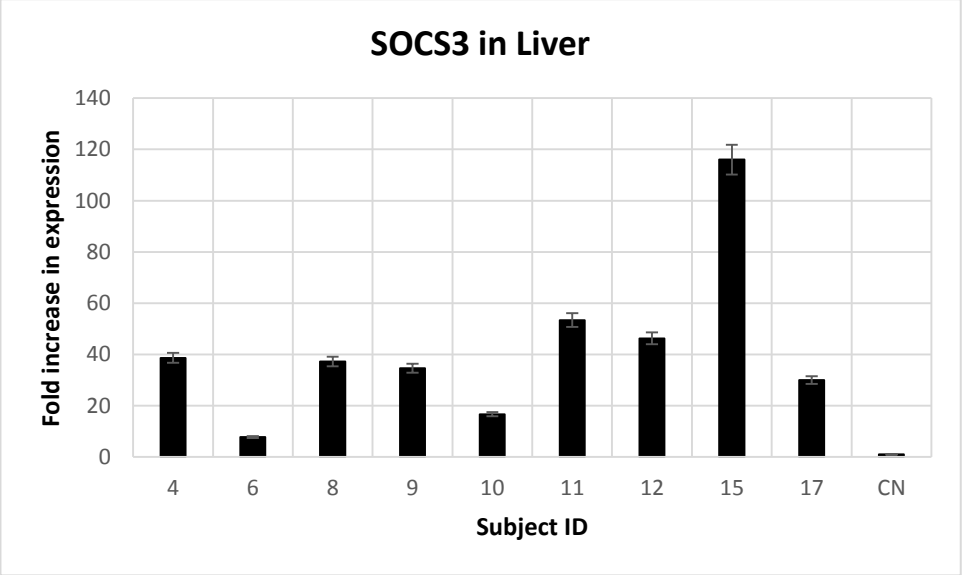

Figure 4.25: SOCS3 expression in the liver by PCR
CN: the average of the 2 samples from the subjects with Crigler-Najjar type 1

In summary, previously, with unsupervised clustering applied on the liver tissue it was only natural that gene transcripts related to the different disease processes would be the ones that would come up as the most variable between groups. When supervised clustering was applied though, and we asked the software to treat this group, which was different to the rest of the patients in terms of muscle and fat, as separate we saw that they were also significantly

different in the liver tissue; they had FOSB isoforms and FOS gene transcripts significantly upregulated.

An analysis was also performed focusing on the 2 more homogenous groups of patients with biliary atresia versus patients with CN type 1. This generated over 200 gene transcripts that were significantly upregulated in the liver of the children with biliary atresia; notably IL8 was one of them (fold change 10.79), but also other genes related to cell growth and fibrosis (e.g. TIMP1, MGP and BTG2).

Chapter 5 INTEGRATED RESULTS AND OUTCOMES

General outcomes

All the patients studied are currently alive. Sixteen of them have had their liver transplant whilst one patient is still awaiting his. The sixteen patients that have been transplanted stayed in hospital after transplantation for a mean of 31 days (SD ±28 days, median 17 days and range 11 to 112 days). During their admission they were ventilated for a mean of 3 days (SD ± 5 days, median 2 days and range 1- 21 days). One of the children suffered repeated episodes of pneumothoraces bilaterally post liver transplant requiring bilateral pleurodesis. Two children suffered from a hepatic artery thrombosis (HAT). One of these children required re-transplantation within a month of his first transplant and the second one required thrombectomy within 24 hours of her transplant. Both children have since made a good recovery. Another child developed post-transplant lymphoproliferative disease (PTLD) requiring treatment with rituximab and a period off immunosuppression. He has made an excellent recovery and he did not require chemotherapy. The table below (Table 5-1) shows the patient IDs and how these correspond the diagnosis and the complications noted after liver transplantation.

Table 5-1: Subject ID with the corresponding diagnosis and the complications noted post liver transplantation

Patient ID	Diagnosis	Complications
1	NSC	Bilateral pneumothoraces
2	BA	No complications
3	BA	PTLD
4	BA	No complications
(5)6	Alagille syndrome	2 admissions, one for diarrhoea and one for fever
7	Re-transplantation	No complications
8	BA	HAT- 2nd liver transplant ACR (2 episodes)
9	A1AT deficiency	No complications
10	BA	No complications
11	BA	HAT , ACR and ventricular bigeminy
12	BA	Biliary complications- required biliary reconstruction
13	Alagille syndrome	1 admission for infection
14	Re-transplantaion	Off waiting list- 3 long bone fractures over 1 year
15	PSC	Stress cardiomyopathy, ACR
16	HCV related cirrhosis	Off waiting list
17	BA	Treatment for CMV reactivation, Leg pains
18	Cryptogenic cirrhosis	Waiting to be transplanted
CN1	Crigler-Najjar type 1	No complications
CN2	Crigler-Najjar type 1	CMV hepatitis

NSC neonatal sclerosing cholangitis, BA biliary atresia, PTLD post-transplant lymphoproliferative disorder, HAT hepatic artery thrombosis, ACR acute cellular rejection, PSC primary sclerosing cholangitis, HCV hepatitis virus C, CMV cytomegalovirus. In bold are the patients whose tissue has been analysed.

Children 1 and 2 live abroad and have not returned since transplant to have their follow up body composition. Patients 6, 7 and 13 are waiting to have their body composition assessment post liver transplant. Patients 14 and 16 are the 2 children that were removed from the waiting list. Patient 16 had a repeat body composition assessment after completing treatment for HCV and patient 14 had a repeat whole body DXA scan. Everyone else who has been transplanted has

had their post liver transplant body composition assessment. At the time of the repeat body composition assessments they were all well in themselves. They all had normal liver function, apart from one patient who subsequently was found to have acute cellular rejection on biopsy the next day. One patient is still waiting to be transplanted.

5.1.1 Body composition and outcomes

Looking at the more basic anthropometry, weight and height sd score did not correlate with days in hospital after liver transplantation, whilst BMI, MUAC and subscapular skinfold sd score had a significant negative correlation with days in hospital after liver transplantation (Pearson correlation factor -0.83 $p<0.05$, -0.76 $p<0.01$ and -0.703 $p<0.05$ respectively).

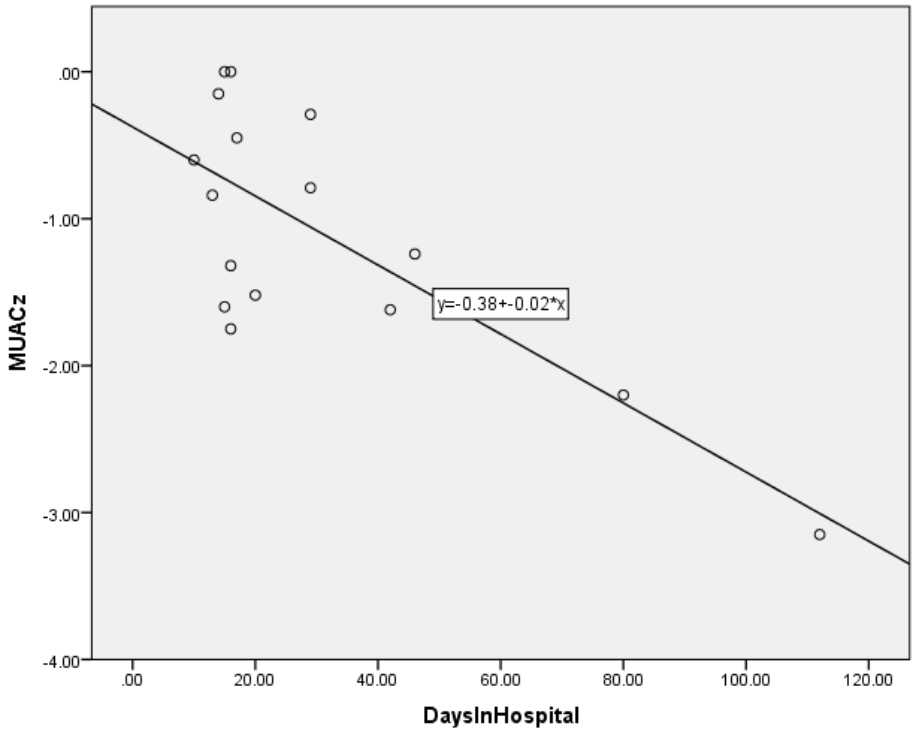

Figure 5.1: Correlation between MUAC and days in hospital after liver transplantation.
MUAC mid upper arm circumference

From the measurements of the DXA scan, none of the FM or FMI were significantly low (i.e. sd score less than -1.96 which is less than the 5th centile). When the patients were separated according to LM and LMI sd scores being less than -1.96 or not, none of the groups were different in terms of length of stay in hospital for any of the indices. Nevertheless there was significant correlation between days spent in hospital after liver transplantation and FM and FMI sd scores for the 8 patients that these were available for. Specifically there were significant

negative correlations between days in hospital after liver transplantation and total FM (-0.78 p<0.05), trunk FM (-0.75 p<0.05), total FMI (-0.859, p<0.01), leg FMI (-0.812, p<0.05), and trunk FMI (-0.907, p<0.01) sd scores. There was no significant correlation between length of stay in hospital and any of the LM or LMI sd scores derived from the whole body DXA scan.

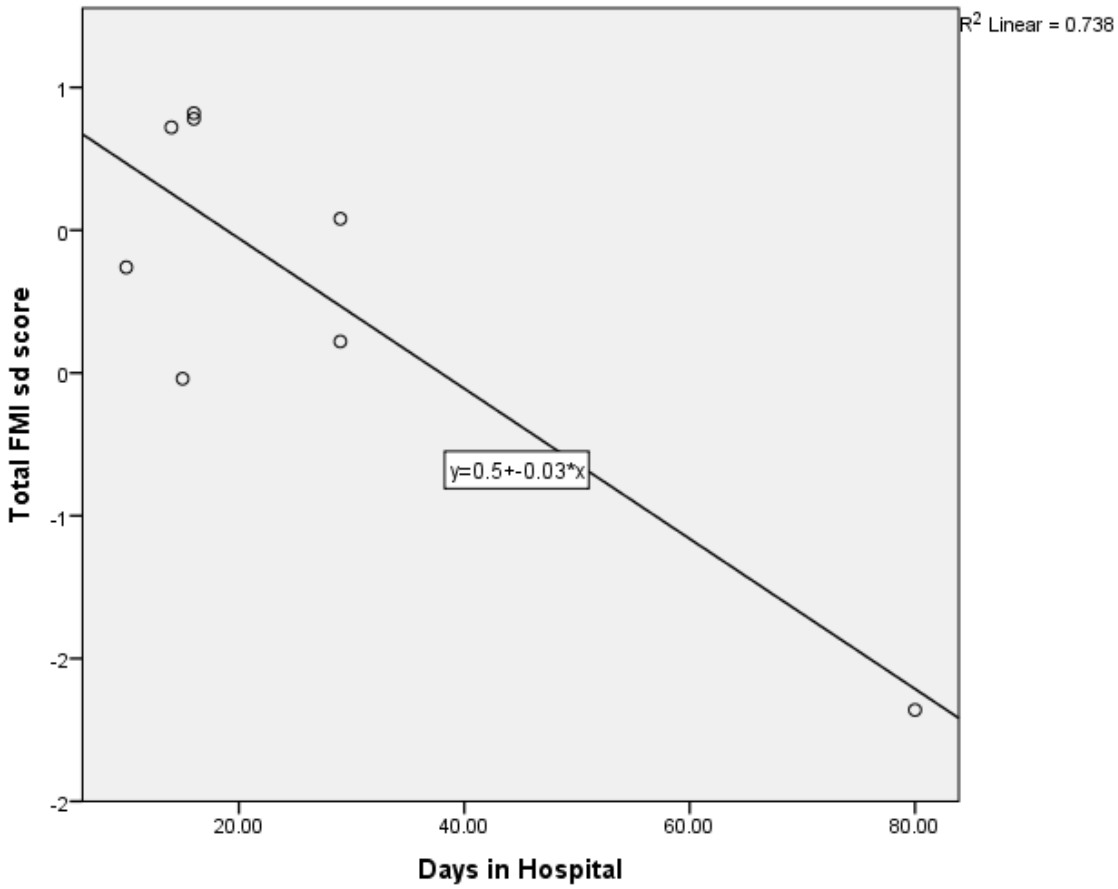

Figure 5.2: Correlation between total FMI sd score and days in hospital after liver transplantation.
FMI fat mass index, sd standard deviation score

The presence of hypermetabolism was not different between the children with ESCLD and the healthy controls. When the patients were separated into 2 groups depending on if they were hypermetabolic or not (when their measured REE was compared against predicted REE) there was no difference in outcomes between the 2 groups.

5.1.2 Body composition and metabolic pathways

The tissue studies highlighted a group of patients as different to the others. This group included patients 3, 8, 9 and 11. All four of these children were Caucasian. Two of them were boys and two were girls. Three of them had biliary atresia and one of the girls had alpha 1 antitrypsin deficiency. As a group their mean stay in hospital after the liver transplant (27.25 days ± 13.94

days) was not significantly different to the mean stay of the other children (38.22 days ± 34.85 days). In regards to post liver transplant complications, the two children who suffered a HAT after their transplant, were both in this group.

The children with the Crigler- Najjar type 1 syndrome were not part of the body composition arm of the study. For these children only weight, height, BMI and MUAC sd scores were available to allow comparisons with the rest of the children. The difference in mean sd score for weight, height and BMI between group 1 (subjects 3, 8, 9 and 11) and group 2 (subjects 1, 4, 6, 10, 12, 15, 17 CN1 and CN2) did not reach statistical significance when tested with an independent t-test. Only the difference in mean BMI sd score approached, but did not achieve, statistical significance with a p score of 0.051.

Table 5-2: Mean weight, height and BMI sd scores for group 1 and group 2

	Mean Weight sd score (±SD)	**Mean Height sd score (±SD)**	**Mean BMI sd score (± SD)**	**MUAC sd score (± SD)**
Group 1	-0.44 (1.09)	-1.18 (1.09)	0.32 (0.98)	-0.9 (0.6)
Group 2	-0.83 (0.73)	-0.54 (1.61)	0.69 (0.67)	-1.3 (1)

BMI body mass index, sd standard deviation score, SD standard deviation, Group 1 includes subjects 3, 8, 9 and 11 and Group 2 includes subject 1, 4, 6, 10, 12, 15, 17, CN1, CN2

When looking at other parameters of body composition, there are no other complete sets of data, so we can only get an indication of what the relationships may be. Looking at the indices from the DXA scan, there are measurements for patients 3 and 11 from group 1 and there are measurements for patients 1, 6, 15 and 17 from Group 2. When the difference of mean sd scores for total FMI, arm FMI, leg FMI and trunk FMI are compared by t-test between these two groups, none of the differences achieve statistical significance. Whereas there are significant differences when comparing the LMI parameters. Group 1 has a higher mean total LMI sd score (1.48 versus -1.49, $p<0.05$), a higher arm LMI sd score (-1.3 versus -2.9, p NS), a higher leg LMI sd score (-0.44 versus -2.68, $p< 0.05$) and a higher trunk LMI sd score (3.61 versus 0.24, $p< 0.05$). There were no significant difference between mean phase angle and mean illness marker from the bioelectrical impedance between the two groups.

Chapter 6 Discussion

The study presented in this thesis was a prospective pilot study and had two parts. The first part involved assessing the body composition of children with end stage chronic liver disease (ESCLD) with various methods before and after liver transplantation. The second part of the study involved studying molecular pathways that are implicated in metabolism in liver, muscle and fat tissue taken from these patients at the time of liver transplantation. The results will also be discussed in two parts in the first instance, with a discussion integrating the results in the end.

Summary of results

The basic anthropometry showed that the patients had a significantly lower weight and height than the healthy controls and this was particularly true for the younger patients. The MUAC was the measurement that was shown to be consistently significantly less for all the patients before transplantation and it showed significant increase after transplantation. The available results from the stable isotope studies showed that total body water of the patients was not different to that of the healthy children. After transplantation there was a reduction in the total body water of the patients, but this did not achieve statistical significance. FM as it can be calculated from TBW was not below 10% in any of the patients before transplantation. There are no available sd scores for values of extra cellular water, but the bromide studies did demonstrate a significant reduction in ECW after liver transplantation.

Similar to the deuterium, the BOD POD also showed that FM was not under 10% of body weight for any of the patients and in fact FM sd scores were all within normal limits. Three of these children had significantly low FFM sd scores (< -1.96) in spite of the BOD POD not differentiating between muscle mass and organ mass. The children with before and after transplant measurements had sd scores for FM and FFM that did not change much and the 2 that had low FFM sd scores before transplant had low scores afterwards as well.

The whole body DXA measurements, done only by the patients as per protocol, showed again that FM and FMI sd scores were within the normal range either when referring to whole body or regional measurements. Six of the eleven patients had some measure of LM < -1.96 sd score either total or regional. When this was corrected for height, 4 of the children had a LMI < 1.96 sd

score. Sd scores tended to remain similar before and after transplantation. DXA correlated the best with the deuterium results.

REE was higher than predicted in 44% of the patients, but also in 50% of the healthy children. RQ was not significantly different between patients and controls. REE/kg after liver transplantation correlated strongly with REE/kg before transplantation.

Moving on to the tissue studies. Microarray analysis of the muscle and fat tissue separated a group of 4 patients from the rest of the patients. These 4 patients, 2 female and 2 male (3 with biliary atresia and one with alpha 1 antitrypsin deficiency) were significantly different to the other patients in regards to the levels of over 400 gene transcripts in their muscle and fat tissue. These gene transcripts included a variety of genes, many of them having to do with inflammation (IL6, CCL2, SELE, SAA1) and extracellular matrix/ tissue regeneration (THBS1, ANKRD1, FMOD) in muscle and also inflammation (IL6, FOS, FOSB, SELE) and protection from oxidative stress (MT1A, EGR1, NAMPT) in fat tissue. From a metabolism point of view the AMPK pathway did not feature in the significantly most variable pathways and, from the evidence we had, its expression in the muscle and fat tissue was not different between the 2 groups of patients, if anything it was most likely down regulated. The metabolic pathway which was shown to be significantly upregulated in this group of 4 patients was the one that included IL6 and SOCS3 and is involved in inflammatory mediated insulin resistance (Wunderlich et al., 2013).

This group of 4 patients' liver tissue (bar patient 3 for whom liver tissue mRNA yield was insufficient) was significantly different to the rest of the liver tissue (bar patient 1 for the same reason) in that FOSB and FOS gene transcripts were significantly upregulated (over 11 and over 4 fold increase respectively). The liver tissue of the children with biliary atresia also had over 200 gene transcripts upregulated in comparison to the 2 children with Crigler-Najjar syndrome type 1. Included in these gene transcripts was IL8 (over 10 fold increase) as well as genes to do with the extracellular matrix (VCAN, MGP, COL4A1).

Overall BMI, MUAC and subscapular skinfold thickness had a significant negative correlation with days in hospital after liver transplantation, as did total and trunk FM and FMI as well as leg FMI, for the patients with DXA measurements. There was no difference in outcomes between the children that were hypermetabolic and those without. The group (patients 3,8,9 and 11) distinguished by the tissue studies as different to the rest of the children, in terms of body composition, had a higher mean BMI sd score which almost reached statistical significance and

significantly higher total, trunk and leg LMI sd scores. There was no difference in the days they spent in hospital after liver transplant in comparison to the other patients.

Body Composition

Body composition in children with ESCLD, as described in the Introduction, is known to be abnormal. In particular, it is known that these children suffer from weight loss, poor growth, muscle wasting, and fat loss and have been thought to be hypermetabolic. In addition, as previously mentioned, assessing these children accurately is challenging, because of the organomegaly and fluid retention.

The results of the body composition studies in this study have confirmed some facts already known about children with ESCLD and have also highlighted some areas of new insight. It is useful to keep in mind that in the modern era children with ESCLD receive intensive dietetic and nutritional input (Henkel and Buchman, 2006). This implies that the changes in their body composition that we see today are in spite of this input. In addition, the group of children studied here were all children considered for liver transplantation. Poor growth and poor nutritional state are important indications for liver transplantation. Therefore these children are not representative of all children with chronic liver disease. They belong to a cohort of children where their chronic liver disease has reached its final stages and liver transplantation is the only means for survival.

Basic anthropometry, weight, height, BMI and measurement of skinfold thickness remains the most accessible bedside means available to assess the nutritional status and progress of these children (Wells et al., 2012). These measurements need to follow certain standards in order for them to be of consistent good quality. Frequently skinfold thickness measurements are thought to be unreliable, because of the variability noted between different people taking the measurements. The advantage in this study was that all measurements were done by the same person.

The anthropometry data presented here highlight that significant differences between the weight and height sd scores of the patients and the healthy controls exist and this was particularly true for the children under 4 years of age. This could be because younger children assessed for liver transplantation are more likely to have more aggressive disease influencing their anthropometry. In fact in spite of weight considered to overestimate the true weight in these patients, the weight sd score of the younger patients was significantly lower than that of the

healthy children. Height sd scores were also significantly lower highlighting the poor growth of these children. This is not an unusual finding in studies of children with chronic liver disease (Chin et al., 1992, van Mourik et al., 2000). MUAC was also shown to be significantly lower in all the patients compared to the healthy children and it increased significantly after liver transplantation. Arm anthropometry has been shown to correlate strongly with FM in healthy children and in children with cystic fibrosis (Chomtho et al., 2006).

The dilution studies, in spite of the initial methodological problems, were able to indicate an increase in total body water through water retention in the patients with ESCLD. When compared to the healthy children the TBW was not different between the 2 groups. From various other methods though, we have seen that at least some of the patients had reduced lean mass. In health, total body water is found in fat free mass. Therefore, if two children have the same TBW, but one has reduced lean mass, this implies that this child has fluid retention. The dilution studies also demonstrated a reduction in the TBW and the ECW after liver transplantation as would be expected with disease related fluid retention. The study by Morgan et al (Morgan et al., 2006) in adults with stable cirrhosis and no overt oedema has shown that on the basis of deuterium dilution studies FM was well above 10% of BW and there was evidence of fluid retention even without obvious oedema. To my knowledge there are no deuterium dilution studies reported on children with liver disease. These results were in accordance with the DXA scan results which did not show a significant increase in lean mass post-transplant (expressed as sd score), and therefore an increase in the TBW post-transplant would not be expected (also expressed as sd score) as a result of increased lean mass.

All patients and healthy controls over 4 years of age had the BOD POD. The BOD POD does require a high degree of co-operation from the children and care must be taken that the children are in the appropriate attire for the measurements to be accurate. All the BOD POD measurements were done following the same protocol and the measurements had to be within narrow limits of agreement, before they were accepted. Only one patient had a significantly low BMI sd score (sd. score below -1.96), but actually her FM and FFM sd scores based on the BOD POD were within the normal range. She was actually a very thin, but also tall girl. Based on the BOD POD, 3 other children were shown to have reduced FFM sd scores, below -1.96. This would be in spite of the presence of organomegaly. When compared to DXA and deuterium dilution studies, BOD POD tended to overestimate fat mass (Wells and Fewtrell, 2006). As fat free mass is derived from the FM, this would lead to an underestimation of the

FFM. BOD POD is designed to measure body volume. BOD POD has been evaluated in healthy children with a variety of body shapes and it has been found accurate when done well (Wells et al., 2011, Wells and Fuller, 2001). Patients with ESCLD often have a particular body shape meaning that the same body volume between a patient with ESCLD and a healthy person does not imply the same composition and therefore the BOD POD equations used to derive the FM may not apply to these patients in the same way that they apply to the healthy children (Dewit et al., 2000). Again to my knowledge there are no air displacement plethysmography studies that have been reported on children with ESCLD.

DXA is designed to measure BMC and BMD. It is clear that our patients had a low whole body bone mineral density. This is in accordance with other studies looking into bone health of children with chronic liver disease (Hogler et al., 2012, Kryskiewicz et al., 2012) and in fact the mean total body BMD of our patients (-1.43) was lower than the mean (-1.0) described in the study by Kryskiewicz et al which includes 18 children. Both studies found vitamin D levels to be unrelated to the BMD. In this study lumbar region DXA was not performed, which is the essential criterion for the diagnosis of osteopenia. DXA measured BMC which was used for the 4C model, but could also be used as a 2C body composition method. DXA had several advantages. It was a simple and quick technique that required minimal co-operation from the patient and with only minimal doses of radiation. It had the added advantage of giving regional data. Concerns have been raised about the accuracy of DXA soft tissue evaluation in the trunk as it involves substantial prediction rather than measurement (Wells and Fewtrell, 2006). This could be of particular relevance to the patients prior to liver transplantation, with relatively distended abdomens, where the measurements of total or trunk LM and FM would not be accurate. The measurements from DXA were also converted to indices in order to remove the effect of height, in a method analogous to that of BMI. The indexed variables were assigned the appropriate sd scores to allow comparisons. Some of these patients were stunted therefore correcting for height gave a more accurate representation of the extent of lean mass or fat loss mass they may have. DXA has not been used to report regional and whole body composition data on children with chronic liver disease, even though it has been used to study BMD. In adult studies, where DXA has been used, differences in FM between cirrhotic patients and controls have been reported mostly as not significant with the exception of trunk FM in females (Riggio et al., 1997), which has been reported reduced.

As with the dilution studies and the BOD POD, so with the DXA, FM remained remarkably within normal limits for the patients. This does not mean that these patients were not in the process of losing fat, but in spite of advanced disease their fat stores appeared preserved and this was true for regional FM (arm, leg and trunk) and remained true when FM was corrected for height as FMI. Even the children who had depleted LM and had very low sd scores for LMI, had FM indices in the normal range. To my knowledge this distinction has not been made before in children with ESCLD or chronic disease in general. Looking closely at the study of Greer et al (Greer et al., 2003) it reports a lower FM of 17% in the children with liver disease in comparison to the 20% of the healthy children, but with no statistical significance between the two means.

As mentioned in the introduction, defining cachexia in adults has had its challenges, but some definitions do exist, whereas in children the situation is more obscure. Defining cachexia as a BMI < 5th centile would mean only one patient being cachectic from our cohort and if the definition was FM < 10% then none of the patients would qualify as cachectic. Cachexia though, as per its definition, is characterised by loss of muscle with or without loss of fat mass (Evans et al., 2008). This definition though applies mostly to patients with cancer, heart failure, COPD etc. and therefore not patients where organomegaly is common. For patients with ESCLD the method which most appropriately assesses appendicular lean mass loss is the whole body DXA. Weight overestimates 'true' weight, because of the water retention and the organomegaly, TBW for the same reasons overestimates lean mass and BOD POD cannot differentiate trunk lean mass from limbs, whereas DXA can provide this valuable differentiation. In fact looking at the DXA results, the trunk LMI indices were all normal or higher than normal most likely because of the organomegaly. If cachexia was defined in our patients as a LMI of either arm or leg below an sd score of -1.96 then 4 of the eleven children who had DXA scans would be cachectic.

The REE results are interesting. When compared to the prediction equations, a significant number of children were hypermetabolic, but actually so were a significant number of the healthy children. The equations used are underestimating the REE. This could be due to differences in the study population and the cohorts used to generate the equations and because of differences in the methodology used to measure the REE. The Henry 2005 equations were based on 166 studies and include data on 10552 people. They are an improvement of the previous Schofield (Schofield, 1985) equations, because they are more contemporary and they have excluded the very large proportion of Italian data in the previous data set used by

Schofield. These equations predict BMR and BMR is measured after a 10-12 hour fast with the subject at rest. REE is any measurement done with a shorter interval of fasting, therefore one would expect REE to be higher than BMR. This also means that when studies report on REE and talk about hypermetabolism by comparing to BMR from prediction equations, one has to be wary of how the REE measured in the study. The advantage in this study was the measurements of the healthy controls. They were done following the same protocol and by the same person and therefore systematic error could be minimised. The study by Carpenter et al (Carpenter et al., 2017) looked into measured REE versus predicted REE in children with end stage liver disease and found that the equations were not suitable of accurate prediction of the children's energy requirements.

Expressing REE per kg of weight helps make comparisons between the different patients. None of the patients had overt ascites and only one was on diuretics, but of course weight is overestimated as there is a degree of fluid retention. Differences in REE/kg were not related to outcomes, but what was striking, was that REE/kg before liver transplant determined REE/kg after liver transplant. This has been shown in adult patients with cirrhosis and it implies that REE/kg depends more on idiosyncratic characteristics of the patients (age, genetics etc.) rather than the disease itself (Muller et al., 1994b). Some authors are in favour of expressing REE per kg weight of fat free mass, as fat free mass is considered the most active tissue from a metabolic point of view. If a patient and a healthy person had the same REE and the same weight, but the patient had a lower FFM, then the patient's ratio of REE/kg of FFM would be higher than that of the healthy person, indicating they are hypermetabolic. There are though some inherent problems with this approach. Fat free mass includes bone, organs and muscle and these elements clearly have a very different metabolic profile and very different energy consumption e.g. muscle contributes 20% to REE whereas the organs 60%. Patients are likely to have a different composition of their FFM, which may not allow a direct comparison with someone healthy. The relationship between FFM and REE is not linear for the full range of FFM and the y-intercept of this relationship is very different to zero. In addition to this measuring FFM in patients frequently comes with its one challenges and inaccuracies. Focusing on a narrower age group of patients with more patients in that group and by including healthy controls, one would be able to make more representative observations for the REE of that age group of patients. Various studies have noted that the presence of hypermetabolism or not is not related to the severity of the liver disease (Ferreira et al., 2014, Prieto-Frias et al., 2016) and have

shown that hypermetabolism after transplantation is correlated to the presence of hypermetabolism before transplantation (Ferreira et al., 2013).

Bioelectrical impedance in spite of being a very easy to use bedside technique did not add much value to the assessment of the patients. BIA machines use equations that are suitable for older patients and the normative data that was available applied to children over the age of 10 years. For BIA to be used more extensively, normative data for the younger children are needed, as are studies of the children who are unwell, so that equations suitable for the latter are generated. Bioelectrical vector analysis (BIVA) would be more useful and could be used for the management of individual patients, but again normative data for children in the UK are required.

3D photonic scanning is a technique that has only relatively recently been used for assessment of body composition. It is not a validated technique. It is though fairly simple for the children to follow and probably the most fun out of all the techniques used in this study. Once this method is validated, it could be easily used to establish more objective anthropometric measurements on the patients (Ng et al., 2016) and certainly the results from this study look promising.

In terms of body composition before and after the liver transplant the mean follow up in this study was 11.4 months. The body composition indices after transplant were strongly influenced by the indices before transplant. There was an indication from the DXA scan that fat mass increased after liver transplantation with a small increase in lean mass. In fact one patient had worse LM indices after transplantation. It may be though that these children need to be studied 5 and 10 years after their transplant or even later as adults in order for body composition changes to be more apparent. As mentioned in the introduction, adult studies confirm an increase in fat mass 1-2 years post liver transplantation.

Looking at the comparison between the various methods, basic anthropometry still has a role, particularly in regards to BMI, MUAC and subscapular skinfold thickness and their correlation with length of stay in hospital after liver transplantation. The advantage in our study in using basic anthropometry was that all the measurement were done by the same person. This wouldn't be feasible in every day practice. If more detailed studies are required then whole body DXA had a good agreement with the 4C model and is a fairly simple, safe and accessible technique to use. One does though need access to appropriate normative data to compare the measurements with. The biggest advantage of the DXA scan is the regional data it may provide. In this study it helped highlight some striking deficits in arm and leg lean mass in our patients.

Deuterium dilution studies are also a feasible option that can be applied for all ages and therefore may generate comparable results. In addition normative data exist. The disadvantage with deuterium dilution is that it is a multi-step procedure requiring precision and consistency throughout for it to be successful. Deuterium also had good agreement with DXA and the 4C model.

Throughout the study fat mass was demonstrated to be in the normal range. Deuterium which was performed on all the children identified 3 children with a relatively low percentage of fat mass, less than 15%. None of the children with valid deuterium results, were had less than 10% fat mass, which would be considered abnormal. Two of these 3 children were old enough to have a DXA scan and their FM indices sd scores were not below -1.96 (< 5th centile), but they did have the lowest FM indices of the cohort. Only 2 of these 3 children with the relatively low fat mass have been transplanted and indeed they had the longest stay in hospital.

Whereas the strength of this work is its prospective nature, the performance of studies not previously done with children with ESCLD and that the various measurements were done by one individual, there are also limitations. The first limitation has to do with the number of patients recruited. This was a pilot study, but there were difficulties in recruiting patients. ESCLD is a rare condition with a limited number of patients on the liver transplant waiting list eligible for recruitment and, in addition to this, the patients also had to live close enough to the 2 sites where the study was done and also to be well enough for a fairly lengthy process of measurements. The most common indication for liver transplantation in children is biliary atresia. The relatively small number of patients studied meant that it was not possible to study patients of only one disease or to have many patients for each different disease. In order to make it possible to study more patients, the ages of the patients covered the whole range for transplantation for chronic liver disease. The relatively small number of patients means that generalisation of the results is limited. One way to address this issue in future studies would be to collaborate with other units and therefore increase the pool of patients.

The patients were assessed once they were on the liver transplant waiting list and of course there was no control as to when their liver transplant would be. This meant that that the patients were assessed at varying stages of their disease journey and of course may have deteriorated further by the time the tissue was collected during transplantation. This is an inherent issue for studies of people on transplant waiting lists and their outcomes (Selberg et al., 1997) . After transplantation the minimum time for follow up body composition measurements was 6 months

and for some it went up to 2 years. This time scale is comparable to other studies that have looked at body composition before and after liver transplantation (Muller et al., 1992b). Again this variation in the timing of the post-transplant measurements hinders comparisons and of course the longer the time the more it allows for changes in height or even lean mass potentially to show. Nevertheless there are very few if any studies of children comparing pre and post liver transplant body composition measurements and one needs to work round the children, particularly the ones that suffer complications after the transplant.

Another limitation of the study had to do with the varying number of children that had valid results for each method of body composition either because of protocol or because of methodological difficulties. This limits of course the comparisons between the various methods but considerable insight has been gained in identifying pitfalls and challenges in applying the various methods accurately and in identifying which methods are most suitable and well received by the patients.

In summary, the findings of this study indicate that in this cohort of children with ESCLD fat mass was relatively preserved and body composition indices related to fat mass were the ones that correlated significantly to length of stay in hospital after liver transplantation.

Tissue Studies

In this thesis microarray validated by qPCR was used to study potential differences in metabolic pathways between patients with ESCLD. Microarray analysis of gene expression in tissue is helpful in identifying patterns that can subsequently guide further experiments. The results from the analysis of liver tissue from 9 children with ESCLD and 2 children with Crigler-Najjar type 1, as well as muscle and fat tissue form 11 children with ESCLD and 2 children with Crigler-Najjar type 1 were summarised at the beginning of this chapter.

In regards to the tissue studies, the obvious limitation is having suitable control tissue to compare the results. This was a unique part of the study that has been done for the first time. As the interest was in metabolic pathways, there had to be patients having similar operations so that systematic error introduced by the type of operation would be minimised. Patients with Crigler-Najjar type 1 are not of course entirely normal, but they are the closest to a normal child from a metabolism point of view having a liver transplant and without of course having ESCLD. We would not expect the Crigler-Najjar patients to be cachectic or to have abnormalities in their

body composition. They did not have their body composition measured, because their involvement in the study was to provide control tissue for the tissue studies.

By using Qlucore Omics Explorer version 3.2 we were able to separate our cohort of children into 2 groups. These 2 groups had differences in the genes expression levels in their various tissue, muscle, fat and liver, and the software was able to help identify the genes that contributed most to this variability. Subsequently the use of GeneGo Metacore™ software helped build networks based on this gene expression variability.

In the muscle tissue it was clear that a subset of the subjects showed an increase in expression levels of IL6 and SOCS3. IL6 is a cytokine with a role in regulating other cytokines with pro-inflammatory and anti-inflammatory properties. IL6 has been described in promoting cachexia and muscle wasting (Chen et al., 2016, Munoz-Canoves et al., 2013), but IL6 is also known as a myokine- a cytokine that can be produced by contracting skeletal muscle after exercise (Pedersen and Febbraio, 2008). There is a lot of controversy round the role of IL6 in insulin resistance. IL6 can be produced by many different types of cells most usually the monocytes and macrophages in response to acute and chronic inflammation (Kreutz et al., 1997), Exercise is accepted to be a way of increasing insulin sensitivity, whilst IL6 is also linked to obesity and insulin resistance (Piya et al., 2013). IL6 produced in skeletal muscle can be produced independently to systemic inflammation and has been linked to low muscle glycogen levels (Keller et al., 2001, Steensberg et al., 2001). IL6 via its receptor pg130, activates JAKs which in turn phosphorylate STAT3 (Xu and Neamati, 2013). This leads to an increased expression of SOCS3 (Starr et al., 1997), which has a role as a negative regulator of IL6 expression.

SOCS3 has some interesting interactions. SOCS3 is implicated in leptin resistance. Leptin binds to a receptor similar, but not the same, to that of IL6 and induces SOCS3 via pSTAT3 (Banks et al., 2000). Cells that overexpress SOCS3 develop leptin resistance and whereas leptin can normally activate the AMPK pathway, in cells where SOCS3 is overexpressed it cannot (Wolsk et al., 2011, Yang et al., 2012). Furthermore, SOCS3 is implicated in insulin resistance by inhibiting IR, IRS1 and IRS2 (Yang et al., 2012).

It has been shown that e.g. after exercise IL6, in a similar way to leptin, mediates improved glucose uptake and insulin sensitivity by activating AMPK without requiring STAT3 (Carey et al., 2006, Watt et al., 2006). It has been suggested that perhaps SOCS3 in conditions of chronic inflammation like in obesity, is able to blunt the ability of IL6 to activate AMPK (Sarvas et al., 2013). In the data presented here, there was indication of increased inflammation. The patients

with the increased expression of IL6 in their skeletal muscle also had increased SAA1 (serum amyloid A1), CCL2, SELE (selectin E is a chemokine which is increased in cytokine stimulated endothelial cells) indicating an environment of inflammation. Perhaps the high levels of SOCS3 in these conditions of inflammation were contributing to a blunted activation of the AMPK pathway by IL6 whereas also inducing insulin resistance with downregulation of the insulin receptors.

Insulin resistance is very well described in patients with ESCLD (Selberg et al., 1993) and in patients with chronic disease in general (Gorjao et al., 2012). The mechanism though of how insulin resistance develops in cirrhosis is not known. Insulin resistance is known to be associated with cachexia (Evans et al., 2008). It is far from clear if it is a coexisting characteristic or if it is actually involved in the mechanism of cachexia. Activation of the AMPK pathway, which is a catabolic pathway, leads to improved insulin sensitivity (Steinberg and Kemp, 2009); this is very well described. It may be that actually insulin resistance is a protective mechanism for the body, particularly in conditions of inflammation, by which it is trying to conserve energy for the inflammatory response.

In regards to the AMPK pathway, in the muscle studied there was not much evidence to support activation of the AMPK pathway. When looking at the expression levels of some relevant genes there was a slight increase in AMPKα1, and a down regulation of GLUT4, PGC1a and an upregulation of FAS. These parameters indicate an overall down-regulation of AMPK. In regards to muscle atrophy, myostatin (MSTN) was down regulated and we found no evidence of increased expression of the atrogenes (MuRF-1 and MAFbx) and therefore no evidence to support increased muscle wasting. The functional studies that are to follow will shed more light on the actual protein expression of the AMPK and the IL6/STAT3/SOCS3 pathways and their potential interaction.

The other highlighted significant networks that came up in the muscle (both with unsupervised and supervised clustering of the sub groups) were linked to pathways related to inflammation mainly through CCL2, cell adhesion interactions and connective tissue degradation. The focus was mainly on the IL6/SOCS3 network, as it is significantly related to metabolism which was the main focus of this study.

In the fat tissue, a similar cytokine profile to the muscle was noted. IL6 remained highly expressed, as did SOCS3. SOCS3 is known to induce insulin resistance in adipose tissue as well (Shi et al., 2006, Yang et al., 2010a). The analysis of the fat tissue also indicated an

increase in PAI-1, a protein coded by the SERPINE1 gene. The primary role of PAI-1 is to inhibit fibrinolysis. PAI-1 is also an acute phase protein and increased levels have been linked to systemic inflammation, obesity, insulin resistance and the metabolic syndrome (Iwaki et al., 2012).

The other networks highlighted as most significant in the fat tissue (either by supervised or unsupervised clustering) involved the NFκB mediated increased expression of IL6, IL8 and CCL2, as well as the IL8 mediated inhibition of PI3K and the upregulation of micro RNA miR-21-5p which has been linked to tumour growth, but is also considered as a marker of endothelial dysfunction and inflammation (Olivieri et al., 2015). Of interest is that IL8 has been associated with inhibition of insulin induced Akt phosphorylation and in this way has been implicated in insulin resistance in adipose tissue (Kobashi et al., 2009).

In regards to the AMPK pathway in particular, the only finding of note was the possible reduced expression of CAMKK2, which is one of the key activators of AMPK. There was also a reduced expression of IRS-1, so again an indication of insulin resistance. Therefore in the fat tissue just like in the muscle, the data indicated an increase in IL6 and SOCS3 and possible insulin resistance, as well as an increase in IL8.

In the liver tissue, when unsupervised clustering was applied, the software divided the subjects into 2 groups, where in one group the patients were the two with the Crigler-Najjar syndrome type 1 and the patient with the Alagille syndrome. All the other patients including the one with the alpha 1 antitrypsin deficiency clustered together in the second group. Of note in this analysis was the significantly increased expression of IL8 seen in the group that included the biliary atresia. The other highly expressed genes mainly had to do with the extracellular matrix and fibrosis (VCAN, TIMP1, CTGF and EFEMP1) except for ACSL4 (an isoform of acyl-coA synthetase that activates long chain fatty acids for either synthesis of lipids or for fatty acid oxidation). Looking at the genes potentially related to the AMPK pathway, AMPKα2 was slightly increased whereas AMPKβ2 was decreased. PFKFB3, a key modulator of glycolysis, was increased and glycogen synthase was reduced, but without knowing the functional state of the protein product of these genes it is not possible to know if the AMPK pathway was activated or not.

When supervised clustering was performed and the patients were separated to those with biliary atresia and to those with Crigler-Najjar type 1 and everyone else was excluded, IL8 was highly expressed in the liver of the children with biliary atresia. This finding is in accordance with

previous reports that IL8 expression is increased in the liver of children with biliary atresia and it is associated with inflammation and fibrosis (Dong and Zheng, 2015).

The muscle and fat data analysis identified the subgroup of subjects 3, 8, 9 and 11 as different to the other subjects. The next step was to see what differentiated this subgroup from the other subjects in regards to their liver, as the liver was the site of the primary disease process. This analysis only included subjects 8, 9, 11 in group 1, as the liver tissue from subject 3 was too small to yield enough RNA and subsequently cDNA to undergo the microarray analysis. The liver tissue from subject 1 had to be excluded for the same reason from group 2. This analysis showed that these 3 patients (subjects 3, 8 and 11) had a significantly higher expression of FOS (4.7 fold increase) and FOSB (2 isoforms 11.04 and 5.77 fold increase) in the liver. FOS and FOSB are 2 of four members of the FOS family. They dimerize with members of the JUN family (c-JUN, JunB and JunD) to form the transcription factor complex AP-1 (activator protein 1). JunB was also significantly increased in the muscle and fat tissue of this same subgroup of patients. AP-1 has a wide variety of roles and is implicated in cancer, fibrosis and inflammatory disorders- it is a known key regulator of hepatocyte differentiation (Stepniak et al., 2006) and it is also involved in inflammation induced insulin resistance (Olefsky and Glass, 2010).

In summary, the results of the tissue studies show with strong statistical significance that the gene transcripts of IL6 and SOCS3 are increased in the muscle and fat tissue of a subgroup of patients in comparison to the rest. From a liver point of view these patients differ in that they express high levels of FOS and FOSB. Taking all the data together one could hypothesize that these children show evidence of inflammatory mediated insulin resistance at a gene expression level. One could hypothesize that the inflammation originates in their liver and then mediates the insulin resistance via cytokines and chemokines in the muscle and adipose tissue possibly via macrophages.

As mentioned when the outcome of these patients was discussed, the tendency was for these four patients to have a higher BMI, more LM and have a shorter stay in hospital after liver transplantation, even though the difference was not statistically significant with the numbers of this study. This seems rather perplexing. The body composition arm of the study indicated a tendency for preservation of fat mass and fat mass was a predictor of stay in hospital after the transplant operation. The field of study where we see inflammation and insulin resistance commonly is in obesity. Of course these patients were not obese, but one could speculate that

in conditions of inflammation, where the body needs energy for the inflammatory response, the body tries to preserve its fat stores, as part of its adaptation mechanism to sickness.

The sickness behaviour of reduced appetite, less movement and insulin resistance could be a way for the body to conserve energy. It is known that, in other situations of increased energy demand, the body has a tendency towards insulin resistance e.g. in puberty (Kelsey and Zeitler, 2016) and in pregnancy (Baeyens et al., 2016). Adult studies in other fields of disease, like coronary heart disease, are reporting that the more obese patients have better outcomes, particularly when there is evidence of higher inflammation (De Schutter et al., 2016). The obesity paradox therefore may not be a paradox at all. It may be part of what the body would try to do anyway in conditions of chronic inflammation and that would be to conserve energy. The development of inflammation mediated insulin resistance may be linked to the conservation of energy and preservation of fat mass.

Chapter 7 Conclusions and Future Work

This study started with some clinical questions. The first question was asking what the body composition and REE of children with ESCLD was before and after liver transplantation. The children with ESCLD studied and reported in this thesis had poor growth, some of them had significant loss of lean mass, particularly in the limbs and they all had relatively preserved fat mass. In addition they had fluid retention and an increase in ECW. Their REE was not increased when compared to healthy children. REE/kg remained similar before and after liver transplant. In regards to body composition after liver transplant, the fluid retention resolved and even though growth parameters improved overall this was mostly due to gain in fat mass.

The second question asked if the children with a higher REE/kg had more complications and worse outcomes post liver transplant and if this increase in REE/kg remained after liver transplantation. The results showed no correlation between REE/kg and length of stay in hospital after liver transplantation or REE/kg and complications post liver transplantation. There was though a significant correlation of pre liver transplant and post liver transplant REE/kg. So in this way REE/kg either higher or lower before transplant persisted after transplant.

The last question asked how the metabolic pathways, that control cell energy metabolism, are affected in children with ESCLD and how do they relate to body composition, resting energy expenditure and outcomes. The analysis performed so far indicates that pathways implicated in inflammation related insulin resistance are upregulated in a subgroup of these patients. These pathways are similar to those implicated in obesity related insulin resistance and it may be that their upregulation is linked to the notable preservation of fat mass demonstrated in these patients. A link between these pathways and REE was not demonstrated. In regards to outcomes there were no clear differences between the two groups of patients. There was some indication that these patients had more lean mass and tended to be discharged home sooner. It is important to note that the only 2 episodes of hepatic artery thrombosis noted in this cohort were in this group of patients.

In conclusion, the children with end stage liver disease of this study had evidence of fluid retention and loss of lean mass whilst their fat mass remained within the normal range. Body composition indices reflecting fat mass had a negative correlation with stay in hospital after liver transplantation i.e. less fat mass correlated with a longer stay in hospital. The children were not hypermetabolic. The tissue studies strongly indicated the higher expression of genes related to

inflammation mediated insulin resistance in a subgroup of these patients and to our knowledge this is the first time this has been shown in children with end stage chronic liver disease.

Future work

There is more work needed to complete this study. In regards to the body composition studies some more children are planned to have their body composition studies performed. Three of the children that have had their liver transplantation are planned to have their repeat post liver transplant body composition in the next 3 months. There is also a child still waiting to be transplanted, who will also need his body composition repeated after his transplant. In addition, three more controls need to have their body composition, they have also been scheduled for the next 3 months.

In regards to the tissue studies, tissue has been collected from children who have had their liver transplants, but they were not in time for the tissue samples to be analysed with the ones presented in this thesis. One additional child with Crigler-Najjar type 1 has been recruited, who has yet to have his liver transplant. These samples, together with the ones from the child still waiting to be transplanted will be analysed in the same way as the ones presented here.

So far from the tissue we have analysed gene expression with microarray and qPCR, but we also need to perform the functional assays. This will be a key point in delineating the pathways studied. E.g. STAT3 is a key regulator of the interaction between IL6 and SOCS3, but it is activated by phosphorylation, so it will be important to see if levels of phosphorylated STAT3 protein are increased in line with the increase in IL6 and SOCS3.

Insulin resistance has been clearly demonstrated in adults with cirrhosis (Selberg et al., 1993), but has not been actually demonstrated in children. The next step to take this work forward would be to measure insulin resistance in children with ESCLD, most likely with a hyperinsulinaemic euglycaemic clamp, whilst also assessing body composition (with whole body DXA and anthropometry) and combining this with tissue studies similar to this work with the addition of a cytokine profile from the children's blood. In this way, one could document the degree of insulin resistance in children with ESCLD and its relationship with fat mass, cachexia and the IL6- SOCS3-IRS1 pathway. This study could then form a basis for longer term follow up of the children into adulthood.

As previously discussed, chronic illness may trigger insulin resistance as a mechanism for the body to preserve energy and it is possible that certain individuals are more prone to this from a

genetic point of view. It is also possible that the individuals that develop insulin resistance during chronic illness may experience epigenetic changes that influence their insulin sensitivity and propensity to accumulate fat and therefore develop the metabolic syndrome increasingly described post transplantation in the future. This hypothesis would be in line with the capacity-load theory for the development of insulin resistance and type 2 diabetes as described from populations that have previously experienced starvation and from children born at low birth weight (Wells, 2017). Being able to define these genetic or epigenetic changes in the future would be helpful in identifying the people at risk of developing metabolic syndrome in the context of chronic illness and this would pave the way in helping to improve their long term outcome particularly after transplantation.

Finally, studying the role of the brain in the development of cachexia and/or inflammatory mediated insulin resistance would have a big impact in this field of research. The brain is likely to have a key role in affecting metabolism, but also is likely to be affected by these perturbations in metabolic profiles. We already know that liver disease at a young age impairs long term neurocognitive outcomes (Kyrana and Dhawan, 2017, Robertson et al., 2013, Lind et al., 2015). As the life expectancy of children with ESCLD has improved dramatically in the last few decades, we will need to gain a clearer understanding of how the brain is involved in this setting of liver dysfunction, chronic inflammation, cachexia and/ or insulin resistance and how these parameters may be manipulated to improve outcomes in a more holistic way for the children in the future.

Chapter 8 PUBLICATIONS

Publications

1) Kyrana E, Briggs S, Dhawan A. Molecular mechanisms of cachexia in chronic disease. Expert Review of Endocrinology and Metabolism, Volume 7, Number 1, January 2012, pp. 73-90(18).

Poster Presentations at Conferences

1) E Kyrana, JE Williams, JCK Wells, A Dhawan. The Importance of Regional Body Composition when using DXA scan in Patients with End Stage Liver Disease (ESLD). ESPGHAN Annual Meeting, Athens 2016.
2) E Kyrana, JE Williams, JCK Wells, RR Mitry, A Dhawan. Four component (4C) body composition and REE in children with end stage liver disease. 8th Cachexia Conference, Paris 2015.
3) E Kyrana, JE Williams, JCK Wells, RR Mitry, A Dhawan. Resting Energy Expenditure (REE) in children with end stage liver disease (ESLD) awaiting liver transplantation. KCL DIIID and DTIMB PhD Research Symposium, London 2015.
4) E Kyrana, JE Williams, JCK Wells, A Dhawan. Four Component (4C) Body Composition in Hepatopulmonary Syndrome (HPS). BSPGHAN Annual Meeting, Stratford upon Avon 2015 and ESPGHAN Annual Meeting, Amsterdam 2015.

Invited Talks

1) Methods of body composition. RSM Paediatric Gastroenterology, Hepatology and Nutrition Study Day, London 2014.
2) Cachexia and models of body composition. BSPGHAN Annual Meeting, London 2014.

Appendix A. List of genes related to AMPK pathway

ACACA
ACACB
ADIPOQ
AKT1
ATG13
ATGL
CAMKK2
CPT1A
CPT1B
FAS
FBXO32
FNDC5
GYS1
HMGCR
IL6
IL6R
IL8
INSR
IRS1
IRS2
LEP
LEPR
LIPE
LKB1
MSTN
MTOR
NFKB1
NFKB2
PFKFB3
PNPLA2
PPARA, PPARG
PPARGC1A
PRKAA1
PRKAA2
PRKAB1
PRKAB2
PRKAG1
PRKAG2
PRKAG3
PYGM
RGS1
RGS2
SAA1
SERPINE 1
SIRT1
SLC2A4
SMAD 2
SMAD3
SOCS3
STAT3
STAT4
STK11
TRIM63
UCP1

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